# Glycosaminoglycans and inflammatory cells in the pathophysiology of neonatal necrotizing enterocolits

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# Contents

**Abstract**

Fulminant neonatal necrotizing enterocolitis (NEC), is characterised by temperature instability, hypotension, disseminated intravascular coagulopathy and oliguria, all clinical manifestations of multi-system organ dysfunction. The capillary leak syndrome (CLS) is common, poses a particularly difficult management problem and is directly and indirectly responsible for many of the complications suffered by infants with this intestinal disorder.

Glycosaminoglycans (GAGs) are key components of the extra-cellular matrix (ECM) and are important for structural stability, the regulation of vascular permeability and trans-endothelial inflammatory cell migration. The capillary leak phenomenon might be explained in part by matrix degradation. The primary aim of this thesis was to examine the nature and distribution of GAGs in NEC. A secondary aim was to describe the inflammatory cell infiltrate (ICI) in relation to GAGs in this disease.

Histochemical experiments were performed on intestine from infants who underwent surgical resection for NEC. GAGs were detected using a cationic gold (CG) method; a 5nm gold conjugated poly-L-lysine probe at

a pH of 1.5 was applied to tissue sections then developed with silver enhancer. Glycanases were employed to determine specific GAG families; sections were incubated with chondroitinase ABC or heparinase III. Control specimens from well-preserved resection margins were incubated with phosphate buffered saline. Matrix and cell surface degradation of GAGs was evident and proportionate to NEC severity. With chondroitinase ABC, GAGs in all layers of the bowel wall were digested, leaving the vascular stain intact. Whilst vascular GAGs were reduced with heparinase III, basement membrane and baso-lateral GAGs were unaffected.

Inflammatory cells produce glycanases, therefore, to explore one possible mechanism of GAG degradation the cellular infiltrate in NEC was examined. The alkaline phosphatase anti-alkaline phosphatase (APAAP) method was employed. Neutrophil elastase, MAC 387, HLA-DR, CD3, CD20 and Ki67 monoclonal antibodies were used. Expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-Selectin was also determined following high temperature citration.

Neutrophils were found to be restricted to the serosa in early NEC, and did not appear in the mucosa in substantial numbers until mucosal ulceration had occurred. The pattern of activation of lymphocytes and up-regulation of adhesion molecules was consistent with an immune response to antigenic stimulation in the early stages of the disease.

The hypothesis was put forward that urinary GAG levels would be elevated in NEC in proportion to disease severity. Urinary GAGs were measured in both an experimental ischaemia / reperfusion (IR) model and in human new-borns with advanced NEC. The primary estimation of GAGs was carried out by standard assay. Alcian blue added to urine reacts with GAGs, forming insoluble complexes. The Alcian blue / GAG complex was dissociated, the dye measured by spectrophotometry and a ratio of GAGs to Creatinine

(GAG/Cr) calculated. Urine from human infants was assayed using a modification of this technique. In addition, the urine from infants was subjected to two-dimensional gel electrophoresis to determine specific GAG subgroups present.

In the experimental model, there was no difference between animals in the IR group and controls. Similarly in human new-borns with

gangrenous bowel secondary to NEC and mechanical intestinal ischaemia, there was no significant difference in the GAG/Cr when compared with controls. However, infants with intestinal ischaemia, and in particular those with confluent intestinal gangrene had abnormal quantities of heparan sulphate (HS) relative to chondroitin sulphate (CS) detected in the urine.

Our immunohistochemical results support evidence of a lymphocyte driven immunopathy as an important part of the mechanisms that underlie disease progression in NEC. Furthermore, in vivo assays of urinary GAGs demonstrated abnormal HS elevation in the presence of gangrenous bowel, suggesting that this may be a useful adjunct in detecting infants with advanced disease.

## Statement of originality

This work is believed to be original and has been carried out by me except where stated. Experiments were performed in the Immunobiology, Biochemistry and Surgery laboratories at the Institute of Child Health, University of London, 30 Guilford Street, London, UK. Further studies took place in the Small Animals Laboratory, Department of Surgery, Groote Schuur, Cape Town and the Department of Biochemistry, Red Cross Children's Hospital, Rondebosch, Cape Town 7700, South Africa.

Histochemical experiments were carried out on material from infants who underwent bowel resection for NEC at Great Ormond Street Children's Hospital, London. A histological score for NEC severity, reported from this department by Sibbons and Spitz was modified by me and applied to histochemical preparations. Quantitative urinary GAG analysis and electrophoresis, on infants with intestinal ischaemia, were carried out by Elizabeth Young, Enzyme laboratory, Institute of Child Health, London.

Intestinal IR experiments were performed by me under the supervision of Prof. S. Cywes, Department of Surgery, Red Cross Children's Hospital, Cape Town. Biochemical tests related to those experiments were carried out by Ruth Brown, Department of Biochemistry, Red Cross Children's Hospital.

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I thank Dr. Nigel Klein for agreeing to be my associate supervisor and for the provision of laboratory space and reagents. I am grateful for what I have learned about the rigours of scientific research and the principles underlying the techniques applied in this project.

I consider myself particularly fortunate to have worked with Professor Lewis Spitz, the principal supervisor of this thesis. His constant motivation, particularly in the final stages, helped to see the work through to completion.

I am indebted to my four parents for the inspiring example that each one has been to me. I thank *awon ara ile* for their, selflessness and willingness to "stand in the gap" in numerous ways. Special gratitude goes to Tosin, my daughter and (not so) little print helper, and to Obafemi, her brother for forbearance during the period of this study.

I owe an enormous debt of gratitude to my wife, Oyinkan, for the innumerable direct and indirect contributions and sacrifices that have made it possible to carry out the work and write up this thesis. I am a fortunate man.

This thesis is dedicated to the memory of Professor Millar Jaja; gentleman and distinguished surgeon.

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# Abbreviations

| | |
|---|---|
| APAAP | Alkaline phosphatase anti-alkaline phosphatase |
| CLS | Capillary leak syndrome |
| CD | Cluster of differentiation |
| CS | Chondroitin sulphate |
| DIC | Disseminated intravascular coagulopathy |
| DS | Dermatan sulphate |
| ECM | Extra-cellular matrix |
| FGF | Fibroblast growth factor |
| GAG | Glycosaminoglycan |
| GAGs | Glycosaminoglycans |
| GAG/Cr | Ratio of (urinary) glycosaminoglycans to creatinine |
| H&E | Haematoxylin and eosin |
| HS | Heparan sulphate |
| HSPG | Heparan sulphate proteoglycan |
| H | Heparin |

| | |
|---|---|
| HUVEC | Human umbilical vein |
| HA | Hyaluronidase |
| IBD | Inflammatory bowel disease |
| ICAM-1 | Intercellular adhesion molecule 1 |
| ICI | Inflammatory cell infiltrate |
| IT | Intestinal tract |
| IR | Ischaemia/reperfusion |
| KS | Keratan sulphate |
| MSOF | Multi-system organ failure |
| NEC | Necrotizing Enterocolitis |
| NICU | Neonatal intensive care unit |
| PBS | Phosphate buffered saline |
| PMN | Polymorphonuclear cells |
| SIRS | Systemic inflammatory response syndrome |
| TCA | T-cryptantigen activation |
| TBS | TRIS buffered saline |
| VCAM-1 | Vascular cell adhesion molecule 1 |

# Publications

Clinical details of infants whose resected specimens have been analysed here and the majority of the work described in Chapters 3 and 4 have previously been published;

**Ade Ajayi N**, Spitz L, Kiely E, Drake D. Resection and primary anastomosis in Necrotizing Enterocolitis. *J R Soc Med* 1996;89:385-388.

**Ade Ajayi N**, Spitz L, Kiely E, Drake D, Klein N. Intestinal Glycosaminoglycans in neonatal necrotizing enterocolitis. Br J Surg 1996;83:415-418.

**Ade Ajayi N,** Spitz L. Mechanisms of glycosaminoglycan degradation in necrotizing enterocolitis. European Surgery; *International Symposium of Pediatric Surgery*, Obergurgl, Tyrol, Austria, January 2003.

# Chapter 1

# Necrotizing enterocolitis and glycosaminoglycans

## 1.1 Introduction

Neonatal necrotizing enterocolitis (NEC) is a common and complex cause of intestinal and multi-system organ dysfunction in the new-born. The capillary leak syndrome (CLS), frequently present in advanced disease, poses a significant management problem in the care of these infants and is associated with a higher mortality (Sonntag et al., 1998).

Glycosaminoglycans (GAGs) are a widely distributed component of the extra-cellular matrix (ECM) and have important roles in structural stability, the regulation of vascular permeability and trans-endothelial inflammatory cell migration (Poole, 1986) (Toole, 1991). The primary aim of this thesis was to examine the relationship between GAGs and the inflammatory cell infiltrate (ICI) in NEC.

Despite considerable research, the aetiology of NEC is unknown. It predominantly affects premature infants and carries a high morbidity and mortality (Stringer and Spitz, 1993). Two groups of babies may be distinguished on clinical grounds. In the first, the course is benign with few constitutional upsets and a recovery that is usually uncomplicated

(Richmond and Mikity, 1975). The majority of deaths occur in the second group of infants who run a fulminant course characterised by major physiological dysfunction including cardiovascular instability, capillary leak and disseminated intra-vascular coagulopathy (DIC) (Bell *et al.*, 1978; Barnard *et al.*, 1985). Multi-system organ failure (MSOF) is often associated with NEC (Morecroft *et al.*, 1994a). The mechanisms underlying the progression to MSOF have not been elucidated. In particular, the CLS is poorly characterised.

GAGs are widely distributed in normal intestine and there is increasing evidence that they have an important role in intestinal homeostasis and maintaining intestinal integrity (Iozzo and Wight, 1982). Degradation of GAGs with resultant protein and fluid loss has been described in intestinal inflammation (Murch *et al.*, 1993). Furthermore, congenital absence of enterocyte GAGs has been associated with extensive intestinal protein loss, producing clinical situations akin to the CLS encountered in NEC and other septic states (Murch *et al.*, 1996; Westphal *et al.*, 2000).

We hypothesised that the pathophysiological processes of NEC result in alterations in the nature and distribution of GAGs. We hypothesised

further that these alterations occur as a result of the activation of inflammatory cells in the diseased bowel. The work presented in this thesis was directed at examining these hypotheses. This introductory chapter will review current understanding of the epidemiology and pathophysiological processes of NEC, emphasising infants presenting with fulminant disease. In addition, the structure function and role of GAGs in intestinal homeostasis will be discussed.

## 1.2 Neonatal necrotizing enterocolitis

### 1.2.1 Historical perspectives

Our current understanding of NEC is based largely on a series of reports from the 1960's and 70's. These coincided with the improved survival of stressed, sick premature infants in newborn nurseries and the wide spread use of premature infant formulas. Berdon described the features now widely recognised as classical for NEC (Berdon et al., 1964). Other reports followed (Mizrahi et al., 1965), (Stevenson et al., 1969), (Hopkins et al., 1970). By 1975, when Frantz reported a 3 year experience of 54 infants with NEC, the group represented 4.7% of all neonatal and 7.3% of all premature infant admissions to the neonatal intensive care unit (NICU) (Frantz et al., 1975). All the infants were formula fed and the overall survival was 35%. The following year Touloukian reported that NEC had become the single most common surgical emergency in the Yale-New Haven Hospital special care unit (Touloukian, 1976).

Historical evidence of the disease pre-dates these reports. Siebold (1825) was reportedly the first to describe perforation of the newborn intestine in

association with infarction (cited by de Sa 1970). In 1891, the German pathologist, Genersich, reported a 2 day old premature baby who died after a 24 hour illness of vomiting and abdominal distension. Post-mortem examination demonstrated ileal inflammation with perforation (Genersich, 1891). Mizrahi also cited Rudner (1915) who described a stillborn neonate with ileal ulceration and perforation (Mizrahi *et al.*, 1965).

Subsequently, reports of neonatal intestinal disease appeared in the literature under different labels. These included, functional obstruction of the newborn, ischaemic and infectious enterocolitis, appendicitis of the newborn and spontaneous gastro-intestinal perforation (Fetterman, 1971). In one such account, Thelander put forward the hypothesis that infection provided an explanation for enterocolitis with perforation in the neonate (Thelander, 1939). It is likely that in this period when it was a poorly defined entity, many neonatal deaths attributed to sepsis and MSOF were the result of unrecognised NEC (Stevenson and Stevenson, 1977). While considerable progress has been made in defining clinical and radiological characteristics of this disorder, the aetiology remains obscure and the mortality and long term morbidity high (Jackman *et al.*, 1990b).

### 1.2.2 The epidemiology of NEC

Necrotizing enterocolitis is widely prevalent and constitutes a substantial part of the workload of neonatal intensive care units around the world. It usually occurs in premature new-born infants recovering from other acute neonatal problems (Kliegman, 1993). Epidemiological data should be interpreted with caution especially when making comparisons between units. Nevertheless, NEC may affect up to 5% of low birth weight babies and over 7.5% of very low birth weight infants (Kliegman, 1993). The increasing incidence appears to mirror improved survival in the premature population. NEC is therefore a world-wide health issue and in some centres, it remains the most common gastro-intestinal surgical emergency in the newborn (Stringer and Spitz, 1993), (Ladd et al., 1998).

Unexplained variations in the local and regional incidence of NEC are common, suggesting that factors related to sub-groups of the local population, peri-natal medical practice or the environment are important. For example, 3 of the 4 major neonatal units in Cape Town, South Africa, have a high incidence of fulminant NEC (Voss et al., 1998), while the remaining unit sees infants with benign or advanced disease infrequently

(Linley, 1998). The same observations have been made in the United Kingdom where surgeons in a major paediatric surgical unit in Southampton are rarely involved in the care of infants with NEC (Wheeler, 1996), while in nearby London, the incidence is substantially higher (Ade-Ajayi et al., 1996a) (Fasoli et al., 1999). In the context of differing responses to the widely prevalent initiating factors of NEC, these regional variations may yet prove important.

### 1.2.3 Risk factors for the development of NEC

NEC represents the end stage of a variety of insults to the affected infant and the aetiology has traditionally been considered multi-factorial. Inter-dependent risk factors were described and a "threshold of injury" hypothesis proposed (Kosloske, 1994a). This suggested that two or more factors acting in concert were required to initiate NEC. In fact, the degree of interaction between risk factors necessary to generate NEC is unclear. Recently it has been suggested that risk factors may represent multiple independent pathways to disease development (Kliegman, 1998).

### 1.2.4 Prematurity

Prematurity is the most consistent feature (Kosloske, 1994a) and is present in over 90 % of infants with NEC. The higher incidence of perinatal asphyxia, respiratory distress and ineffective auto-regulation in this group of infants explains the need for invasive medical interventions. These combinations may predispose to NEC by producing a small, sick and stressed infant (Milner et al., 1986). Associated factors of pathophysiological significance include gastro-intestinal immaturity with mucosal permeability (Israel, 1994), dysmotility and humoral (IgA) deficiency (Vasan and Gotoff, 1994). The susceptibility of low birth weight animals to the development of a disease with many of the features of NEC has been confirmed experimentally (Sibbons et al., 1988).

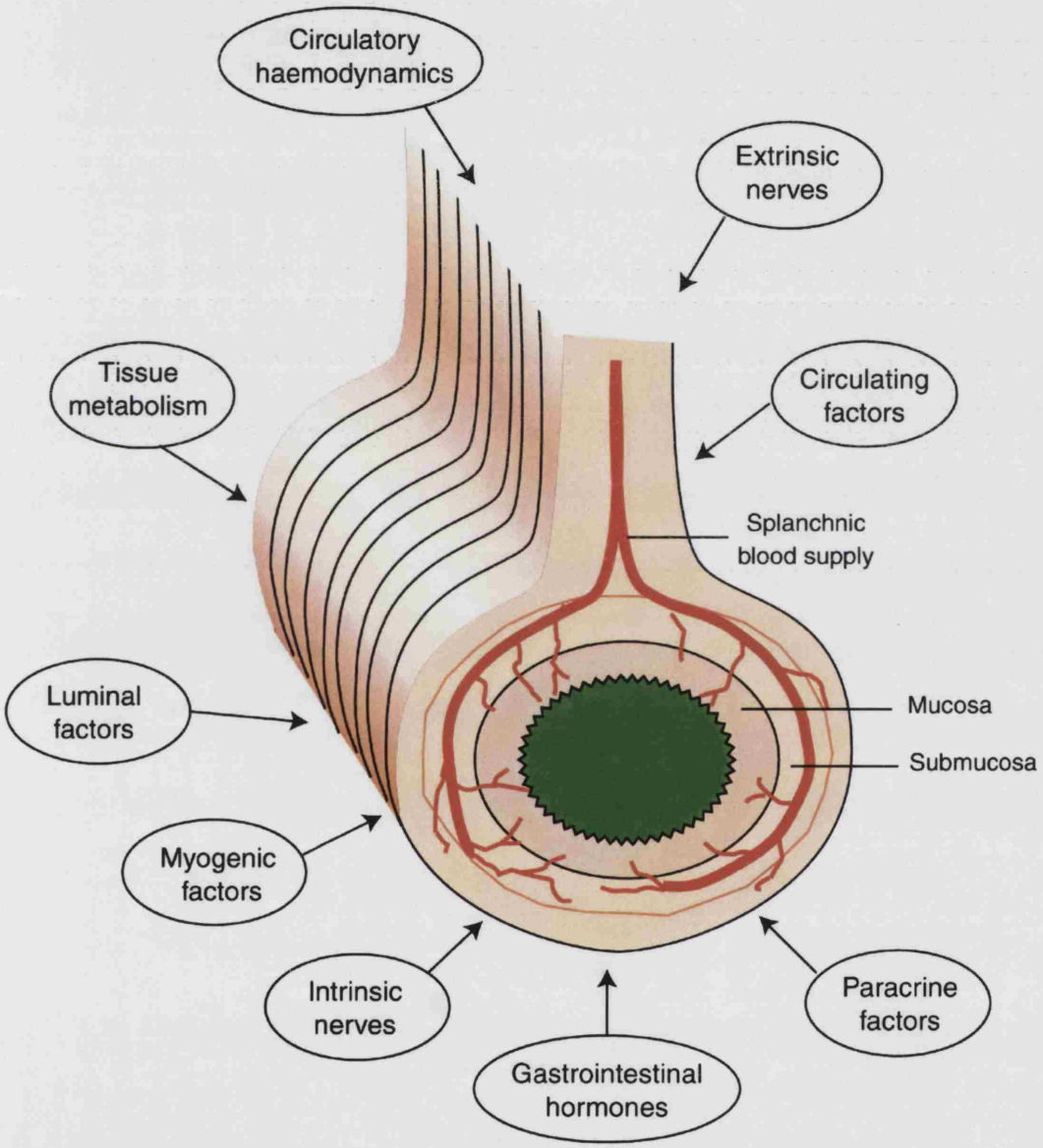
### 1.2.5 Impaired splanchnic perfusion and hypoxia

Intestinal blood flow in the new-born is subject to complex regulatory mechanisms (see Figure 1). Mesenteric oxygen and perfusion demands are high, and are determined by baseline intestinal cellular and metabolic activity, nutritional requirements, motility and gastro-intestinal growth.

The potential to respond to these demands is limited by gestational age and is mediated by autonomic and peripheral nervous systems as well as autocrine and endocrine control mechanisms (Kliegman, 1998).

In 1939, Thelander implicated perinatal hypoxia as a contributor to infant sepsis (Thelander, 1939) and in 1969, Lloyd popularised the concept of the diving reflex during episodes of systemic hypoxia or hypoperfusion (Lloyd, 1969). This hypothesis of selective mucosal ischaemia was based on work by Scholander who demonstrated bradycardia with the maintenance of blood pressure in pearl divers (Scholander et al., 1962). Despite alternative views, the diving reflex theory with intermittent mucosal ischaemia alternating with improved perfusion, remains an attractive hypothesis to explain the observation of patchy ischaemia/reperfusion (IR) injury observed histologically in infants with NEC (Joshi et al., 1973, Joshi, 1978). The hypothesis is supported by experimental work, including that of Touloukian who demonstrated that asphyxiation of neonatal piglets resulted in mucosal ischaemia, secondary to selective intestinal hypoperfusion. (Touloukian et al., 1972)

**Figure 1** Complex mechanisms regulating intestinal perfusion.




*Adapted from Parks 1985*

A range of other conditions predispose to NEC by reducing oxygen carrying capacity or mucosal perfusion. These include congenital heart disease, umbilical artery catheterisation (Cochran et al., 1968), (Williams et al., 1972, Neal et al., 1972), polycythaemia and exchange blood transfusions (Friedman et al., 1970) (Guaran et al., 1992) (Corkery et al., 1968). Initially, the infant is able to compensate for the changes, but as the reduced oxygenation becomes more severe, the compensatory mechanisms are exhausted and alternative energy substrates are utilised, resulting in a sequence of adverse events (Nowicki and Nankervis, 1994). Understanding the mechanisms that initiate theses adverse events may result in novel and efficacious adjunctive interventions for NEC.

### 1.2.6 Intra-luminal injury

Mucosal injury by luminal contents constitutes another risk factor for NEC. That the majority of infants have been fed prior to the development of NEC is well documented (Kosloske, 1994a). That upwards of 90% of those fed have been fully or partially fed formula milk is also well reported. What is lacking is scientific data, directly linking formula feeds with the development of NEC.

### 1.2.6.1 Cow's milk predisposes to NEC

Intolerance to formula milk has been implicated in several observational and anecdotal studies (Book et al., 1976) (Schanler et al., 1999) (Stein, 1977). A number of pathophysiological mechanisms have been put forward to explain this. One example is that based on the immaturity of motor function and lactase activity in the premature intestine. This results in stasis and an inability to digest the lactose present in infant formula milk. This may contribute to the development of NEC, as a result of bacterial fermentation of excessive carbohydrate to short chain fatty acids with a resultant drop in luminal pH.

Epidemiological data in support of the importance of breast milk in preventing NEC was provided by Lucas in 1990. In a prospective multi-centre trial involving 926 premature infants, formula and breast milk fed babies were followed for the development of NEC (Lucas and Cole, 1990). Overall, infants fed formula milk alone developed NEC 6-10 times more commonly than those on breast milk alone. Partial breast milk feeding was also found to be protective. Furthermore, babies with a gestational age greater than 30 weeks whose diet included breast milk rarely developed the

disease. In formula only infants of this group, NEC was 20 times more common, strongly suggesting that breast milk plays an important role in NEC prophylaxis. It has been suggested that the benefits of breast feeding may in part reflect it's anti-inflammatory properties (Buescher, 1994).

### 1.2.6.2 Feed volumes and advancement

Large feed volumes and rapid feed advancement have been implicated in a number of reports including that of Anderson (Anderson and Kliegman, 1991). The physiological explanation for this is an inability by the stressed premature infant to respond to the increased nutritional demand with an adequate increase in mesenteric blood flow. Despite what appears to be a logical hypothesis, prospective randomised studies have failed to demonstrate a consistent adverse effect to early commencement of feeds (Ostertag et al., 1986), large volume feeds and rapid feed advancement (Rayyis et al., 1999). Furthermore, a delay in introducing enteral feeds does not appear to reduce the incidence of the disease and may in fact increase it (LaGamma et al., 1985). Until more data becomes available, it seems appropriate to take a moderate approach to feed commencement, volumes and rates of increase.

### 1.2.6.3 Hyperosmolar luminal contents predispose to NEC

In addition to the type and volume of feed, the concentration is thought to have a bearing on the development of NEC. Histological evidence to support the hypothesis that hypertonic feeds are responsible for intestinal mucosal injury was provided by Kameda who utilised different concentrations of a variety of solutions and produced villous injury in rat small intestine (Kameda et al., 1968). In another study, goats colonised with E Coli and then fed hyperosmolar goat milk underwent mucosal necrosis (De Lemos et al., 1974).

Hyper-osmolar feeds, common in the 1960's and 70's have largely been phased out. However, hyper-osmolar medication may unwittingly be administered to infants, contributing to the development of NEC. Vitamin E, xanthine derivatives, calcium lactate and Indomethacin are among drugs that have been implicated in this regard (Willis et al., 1977) (Mutz and Obladen, 1985).

### 1.2.7 Microbiological profiles in NEC

It has not been possible to demonstrate a single causative agent for NEC but the microbial theory is strengthened by the occurrence of clustering. Engel suggested a link between *Clostridium perfringens* and NEC (Engel, 1974) and a number of other authors have reported similar findings (Kliegman, 1979). Other organisms implicated include *Escherichia coli* and *Klebsiella spp* (Speer et al., 1976). In one study, Virnig reported 5 infants presenting in a 19 day period with NEC (Virnig and Reynolds, 1974). *Escherichia coli* was the commonly cultured organism. Kosloske described 17 infants with NEC. Seven of the ones with the most aggressive course had *Clostridia* isolated. Of this number, 4 had *Clostridium perfringens* and all ran a fulminant clinical course. The remainder either grew *Klebsiella, Staphylococcus aureus, Streptococcus faecalis* or *Bacteroides*, or had negative cultures. In a follow up to this study, the same authors obtained blood cultures and peritoneal fluid in 25 infants with NEC. Seventeen required surgery and all but 1, had bacteria isolated from blood and/or peritoneal fluid. None of the 8 infants who ran a benign course and were treated medically had a positive growth from peritoneal fluid culture and only 2 had positive blood cultures (Kosloske and Ulrich,

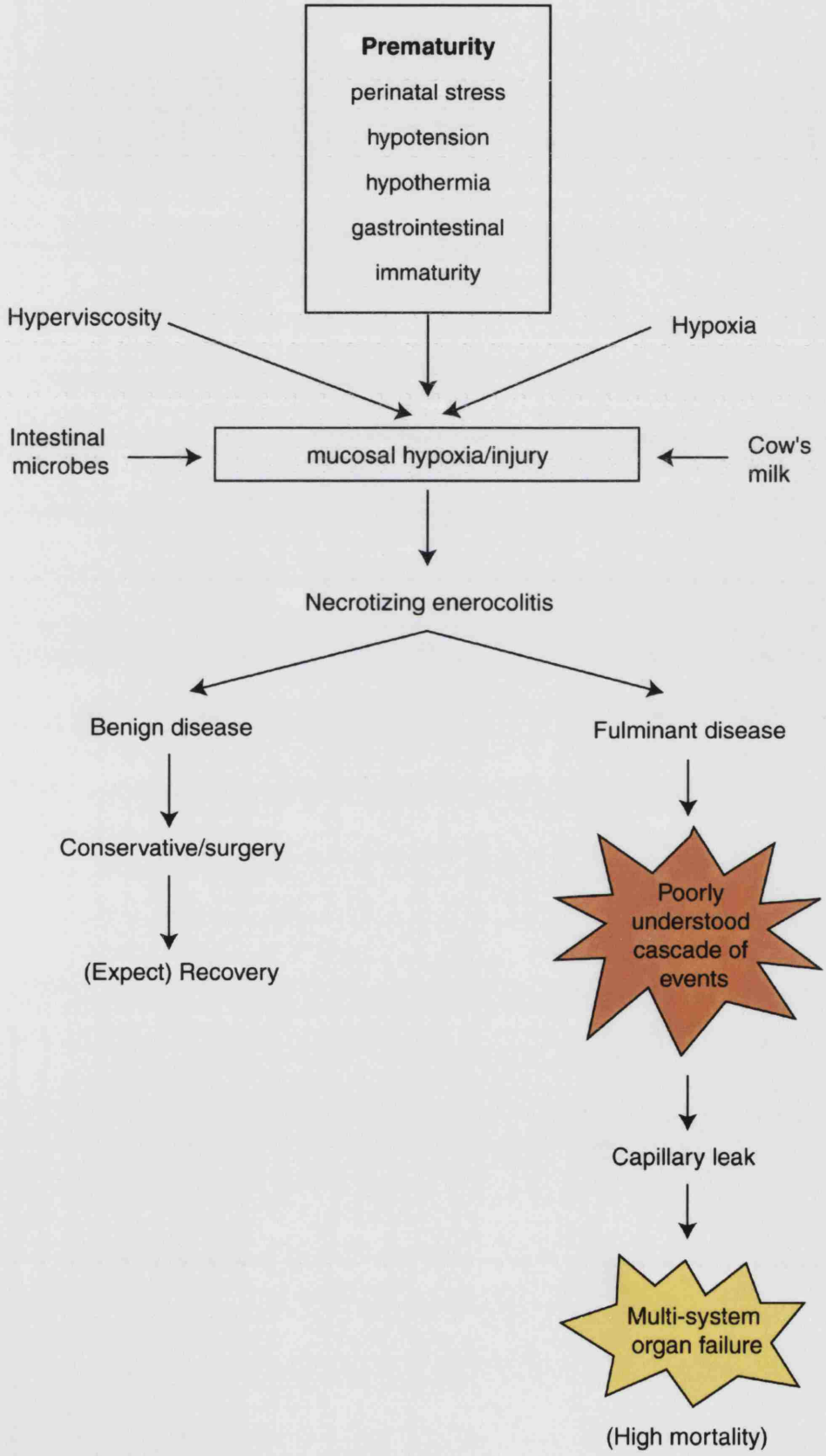
1980). Outbreaks of NEC secondary to viruses have also been reported (Rotbart et al., 1988) and infection control measures to reduce the transmission of putative transmissible agents, reduce the likelihood of developing NEC (Book et al., 1977).

It is of note that despite these and other well-defined clusters of NEC associated with the isolation of a microbial agent, the majority of cases cannot be so categorised. One possible explanation is that products of microbial agents rather than the microbes themselves are responsible for the initiation of NEC (Kliegman, 1998).

### 1.2.8 Summary of risk factors

Despite extensive investigation the aetiology of NEC remains unclear. There are, however, risk factors including prematurity, low birthweight, hypoxia, intestinal ischaemia, hyperosmolar feeding and mucosal invasion by microbes. A diagrammatic summary of risk factors and their interactions is given in Figure 2.

**Figure 2** Summary of the interactions between the major risk factors that initiate neonatal necrotizing enterocolitis.



### 1.2.9 Experimental models of NEC

There is no consensus regarding the optimum experimental model for NEC (Sibbons et al., 1997). Central to this controversy is the question of whether risk factors are inter-dependent or independent disease initiators (Kliegman 1998). Is it more important to reproduce the initiating risk factor(s) or to have end points in which physiological and histological features consistent with the human disease are achieved? The result of the debate is that a large number of models have been proposed for NEC (Sibbons et al., 1988) (De Lemos et al., 1974) (Sibbons et al., 1997).

The triad of prematurity, intestinal ischaemia – hypoxia and intra-luminal injury as described above are the major groups of risk factors. The majority of models proposed for NEC have presumed the independent ability of one of the risk factors described above, to generate the sequence of events leading to NEC. Those emphasising the ischaemia – hypoxia theory include the use of ischaemia with or without reperfusion, hypoxia, controlled haemorrhage, hypothermia, exchange transfusions and combinations of the above (Sibbons et al., 1997). Intestinal IR is one of the most widely used models. It has the advantage of being reproducible,

relatively inexpensive and it appears to produce both the physiological derangements and histopathological changes similar to the human disease.

Luminal insults have been produced in a variety of ways such as by the instillation of hyperosmolar solutions (eg acidified Casein), and microbial agents and their products including *Clostridium perfringens*, *Escherichia coli*, *Klebsiella spp.*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacteroides* and lipopolysaccharide (Sibbons *et al.*, 1997). The above models have also been tried in various combinations in the attempt to fully reproduce the features of NEC. The best available model for NEC combines prematurity, luminal hyperosmolality and intestinal hypoxia.

### 1.2.10 The diagnosis of NEC

#### 1.2.10.1 Clinical

A spectrum of disease from mild malaise to fulminant NEC is recognised. Clinical presentation may be with lethargy, bilious vomiting, abdominal distension and blood in the stools (Harms *et al.*, 1994; Dudgeon *et al.*, 1973). A palpable abdominal mass and erythema of the anterior abdominal wall may be evidence of underlying intestinal gangrene in an otherwise stable infant. In fulminant disease, in addition to the above, NEC may present with, cardio-vascular instability, capillary leak, collapse and a

necrotizing process which may extend from the intestine to involve surrounding tissues including the anterior abdominal wall (Figure 3). The majority of deaths occur in this second group of infants.

Bell proposed the now widely used criteria for the diagnosis and classification of infants with NEC (Bell et al., 1978). Lethargy, feed intolerance and bile stained vomiting characterise stage I and the presence of pneumatosis intestinalis (PI) is the hallmark of stage II disease. Stage III NEC is associated with septic shock, perforation and life-threatening haemorrhage. Bell's original criteria have been modified in an attempt to reflect the heterogeneity of the condition (Walsh and Kliegman, 1986).

**Figure 3** Necrotizing fascitis as a complication of fulminant neonatal necrotizing enterocolitis.

### 1.2.10.2 Radiological

A number of imaging modalities including abdominal ultrasound may have a role to play in the diagnosis of NEC but, in the clinical context described above, plain abdominal radiography remains the gold standard. The presence of dilated bowel loops with thickened walls and pneumatosis intestinalis (PI), is the hallmark of NEC (Bell et al., 1978). This tramline appearance is the radiological reflection of gas within the intestinal wall (Figure 4). PI is present in 50 - 90% of cases of NEC but may be absent in established NEC (Pochaczevsky and Kassner, 1971). In addition, PI may be present in sick infants with other conditions such as bowel infarction from mesenteric ischaemia secondary to volvulus. The distribution of PI may be limited or extensive. Portal venous gas may also be demonstrated within the liver on abdominal radiography. This is considered by some to represent fulminant disease, but it's significance is debatable (Vollman et al., 1976). Pneumoperitoneum is widely considered the only absolute indication for surgical intervention. Even when this is present, the signs may be subtle and open to observer interpretation. In addition Rabinowitz has stated that up to half of all intestinal perforations are simply not apparent on abdominal x-ray (Rabinowitz and Siegle, 1976) .

**Figure 4** Pneumatosis intestinalis; the radiological hallmark of NEC. This tramline appearance (arrow) is the reflection of gas within the intestinal wall.

### 1.2.10.3 Haematological and biochemical

Efforts to predict the likelihood of surgery and the outcome of individual infants at presentation with NEC are fraught with difficulty, but some useful parameters have emerged. Dykes retrospectively analysed 27 factors in 80 neonates with NEC and identified 3 of prognostic value including blood pH and platelet count (Dykes et al., 1985). This enabled the calculation of a probability of death score. While the pH and platelet count at presentation are now widely used, it has been argued that disease severity scores contribute little to individual patient management. Ultimately, a better understanding of basic pathophysiological mechanisms which will result in individualised treatment is what is required.

More recently, there has been interest in the prognostic value of the Thomsen-Freidenreich (T) cryptantigen in NEC. It occurs naturally on the surface of human red blood cells and is usually covered by a layer of N-acetylneuraminic acid (Kirsten et al., 1996). Cleavage of N-acetylneuraminic acid by neuraminidase, a product of gram negative bacteria, exposes the underlying antigen. T-cryptantigen activation (TCA) renders red cells susceptible to haemolysis following the transfusion of

blood products containing anti-T antibodies (Squire et al., 1992). In observational studies, TCA has been associated with advanced NEC and a higher mortality (Klein et al., 1986) (Osborn et al., 1999).

Whether TCA will prove to be a consistent marker of fulminant disease awaits wider validation. In a recent 10 year review, one quarter of infants with NEC were TCA positive and 96% of these had stage III disease. The laparotomy rate of TCA positive infants was higher, and widespread intestinal gangrene more common than in the negative group (Hall et al., 2001).

#### 1.2.10.4 Novel techniques

Novel techniques for early detection of NEC include the use of hydrogen breath tests and recto-sigmoid pH monitoring (Koivusalo et al., 2000). The clinical usefulness of these techniques awaits further assessment.

### 1.2.11 Management of neonatal necrotizing enterocolitis

The principles underlying the conservative management of NEC are widely agreed and have changed little in 30 years. In 1973, reporting 14 infants without perforation of a total of 23 who had NEC, Bell recommended a medical strategy to reduce the number of infants proceeding to perforation. This involved naso-gastric decompression, parenteral and gavage antibiotics and general support of the infant (Bell et al., 1973). Intravenous steroids have been used in the past as adjunctive treatment in haemodynamically unstable infants with NEC on the grounds that their physiological derangement reflected endotoxaemia (Brown and Sweet, 1980). This approach is no longer widely used.

Currently, a typical NEC treatment protocol includes, a naso-gastric tube for gastric decompression, parenteral nutrition and broad-spectrum antibiotics intravenously. The baby receives intravenous antibiotics for 5-7 days and is left fasting for 10 days. The evidence for this as the best treatment regime comes from a report by Frantz et al in 1975. A mandatory 10 day period of fasting was recommended as in several patients in whom enteric feeding began before 10 days, clinical and radiological signs of

NEC reappeared. There are no prospective studies in support of this 10 day rule. For those patients who deteriorate despite medical treatment, surgical intervention is usually indicated.

Up to 50% of babies with NEC may develop advanced disease and require surgical intervention (Kosloske, 1985). The indications for surgical intervention and the most appropriate procedure remain controversial. Pneumoperitoneum is widely regarded as an absolute indication for surgery. Gross abdominal distension, cardiovascular instability, failure of medical management as evidenced by persistent acidosis, the presence of a palpable mass and a fixed loop on repeated plain abdominal x-rays all constitute relative indications. Many authors consider the ideal time for intervention to be following the development of necrosis but prior to intestinal perforation (Kosloske, 1994b).

Typical features at laparotomy include gangrene and perforation. The disease may be isolated, segmental or multi-focal, findings that have a profound influence on the surgical decision-making, and may be reflected in the histopathology. The principles of surgery for NEC are urgent decompression if distension compromises breathing or ventilation,

peritoneal toileting, resection of bowel that is unquestionably gangrenous with preservation of bowel that is normal or whose viability cannot be ascertained. Specific surgical options currently available include peritoneal drainage under local anaesthetic, enterostomy only, resection and enterostomies, resection clip and drop and resection with primary anastomosis (Harberg et al., 1983) (Ade-Ajayi et al., 1996a) (Stringer et al., 1993) (Ein et al., 1990) (Ricketts, 1994).

### 1.2.12 Prevention of NEC

Strategies employed to reduce the incidence of NEC include steroid induction of intestinal maturation, prophylactic antibiotic gavage, oral immunoglobulin administration, modification of enteral feeds, and the manipulation of intestinal bacterial flora (Vasan and Gotoff, 1994) (Egan et al., 1976) (Hoyos, 1999).

The administration of antenatal steroids to mothers in premature labour is now routine in many obstetric units. This is given primarily to induce surfactant formation in the premature lung but is considered by some to have the added benefit of increasing intestinal maturation and possibly reducing the incidence of NEC. Another intervention used for prophylaxis

is the administration of oral immunoglobulin. Although its efficacy in preventing NEC in low-birth-weight infants has been demonstrated in clinical trials (Eibl et al., 1988), it has not gained widespread acceptance.

Barlow demonstrated the protective effects of breast feeds in newborn rats subjected to hypoxia (Barlow et al., 1974), a finding, which as suggested above may reflect the anti-inflammatory properties of breast milk. It may be that the single factor, amenable to medical and parental manipulation, and providing effective prophylaxis to NEC is feeding. The widespread use of breast milk feeds with moderate volumes and increments in the at risk population may reduce the incidence of this disease.

### 1.2.13 Pathological findings in NEC

Gross pathological findings in NEC range from isolated ischaemic change with or without perforations to extensive multi-focal disease or confluent gangrene. Histopathological features include haemorrhage, oedema, ulceration, evidence of inflammation and necrosis (Stevenson et al., 1969). The infiltrate consists of neutrophils, lymphocytes, plasma cells, histiocytes and mast cells (Tait and Kealy, 1979), but has not been fully characterised in terms of specific distribution and recruitment patterns.

### 1.2.14 Necrotizing enterocolitis and glycosaminoglycans

Despite the advances in general neonatal care and improvements in surgical instrumentation and expertise, the complications following surgical treatment of NEC remain high. Laparotomy carries a mortality of 20-50% (Stringer et al., 1993), (Kosloske, 1985), (Ade-Ajayi et al., 1996a). and half of the survivors may suffer long term morbidity including neuro-developmental delay and the short bowel syndrome (Jackman et al., 1990a).

There is a correlation between NEC severity and the incidence of complications (Walsh et al., 1989). The CLS which is a feature of fulminant NEC is associated with a higher mortality (Sonntag et al., 1998). Modulation of GAGs has been shown to result in CLS in other septic states and we sought to investigate the potential role of GAGs in NEC. An introduction to the structure and functions of GAGs is provided in the next section.

## 1.3 The structure and function of glycosaminoglycans

### 1.3.1 Introduction

The extra-cellular matrix (ECM) is widely distributed between cells in living multi-cellular organisms. In this thesis, the term is used in its broad sense to refer to both cell surface and matrix molecules. Once considered homogenous in nature and structural in function, adequate evidence now exists that the ECM consists of a complex and important group of substances involved in multiple interactions with wide ranging effects (Poole, 1986) (Toole, 1991).

Collagen and elastin are two of the major proteins of the ECM and form the fibrous elements of the matrix. They exist within a non-fibrous groundwork of glycoproteins and proteoglycans whose components include laminin, fibronectin and glycosaminoglycans (GAGs). Within the intestinal wall, prominent distribution sites of the ECM include apical and baso-lateral epithelial surfaces, basement membrane, the sub-mucosa and muscular layers. At these sites, ECM components function to provide structural support, act as substrates for cell adhesion and migration,

regulate permeability across membranes, bind growth factors and bacteria and are involved in the regulation of cellular differentiation (Ishai-Michaeli et al., 1990) (Isaacs, 1994) (Toole, 1991) (Aota and Yamada, 1994). Many of the interactive properties of ECM components are determined by cellular receptors called integrins.

Enterocytes and colonocytes are anchored to the basement membrane and to one another. Interest in the role of the ECM responsible for this structural function in the intestine is rising (Vantrappen and Geboes, 1993). Current knowledge is based largely on experimental animal work and a few adult reports (Kedinger et al., 1986). There are few published reports of the distribution of ECM components in human infant bowel and no reports on the fate of the ECM in bowel affected by NEC.

### 1.3.2 General structure of GAGs

Glycosaminoglycans consist of disaccharides (usually an amino-sugar and a uronic acid residue) in repeating sequence forming unbranched polysaccharides. The amino sugar is either N-acetyl glucosamine or N-acetyl galactosamine while the uronic acid residue is D-glucuronic acid or L-iduronic acid.

GAGs are differentiated on the basis of their sugar constituents, linkage types and degree of sulphation (Toole, 1991). They range from 6,000 to several million in relative molecular mass. Highly anionic, they have an affinity for cations such as sodium with the result that they are hydrophilic and relatively bulky in terms of spatial relations. Specific glycosaminoglycan (GAG) families are hyaluronidase (HA), chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparan sulphate (HS) and heparin (H) (Toole, 1991).

All GAGs with the exception of HA are significantly sulphated (Lindahl et al., 1977). Apart from HA and H, GAG chains are bound covalently via an O-glycosidic link at the reducing end to a serine residue or via an N-link to asparagine in a core protein. A variety of macromolecules, collectively known as proteoglycans, is the end result (Poole, 1986). Proteoglycans (PGs) are found within and on cell surfaces and in the extra-cellular matrix (Wight et al., 1991). Their structural diversity explains the wide range of biological functions attributed to them.

### 1.3.3 Synthesis and degradation of proteoglycans

Synthesis of the core protein takes place in the endoplasmic reticulum. This is followed by the organised addition of oligosaccharides and sulphate to the non-reducing ends of these chains, a process that takes place in golgi bodies (Silbert, 1982). Purified peptide growth factors, insulin and ascorbic acid have all been shown to have a stimulatory effect on PG synthesis (Prins *et al.*, 1982). Physiological degradation of PGs commences with the cleavage of core proteins in extra-cellular sites followed by the intra-cellular digestion of GAGs (Silbert, 1982). With the exception of HA, the properties of GAGs are dependent on their core proteins. For the purposes of this thesis, GAG sub-groups will be discussed as though they are distinct entities.

### 1.3.4 General properties of GAGs

The general properties of GAGs include vascular permeability, cell to cell adhesion, signal transduction (Binari *et al.*, 1997), protein interactions, ECM assembly (Schaefer *et al.*, 1996; Chung and Erickson, 1997),

mediation of microbial interactions (Isaacs, 1994) and structural stability (Poole, 1986).

Vascular permeability to macromolecules is related to size and electrostatic charge. The surface charge of endothelium regulates the movement of molecules from one compartment to the other. For example albumin clearance through the kidney is considerably less than that of similarly sized but neutral molecules (Kanwar, 1984). The endothelial surface charge is determined by its GAG content. Kanwar has also demonstrated the passage of larger moieties through the glomerular basement membrane of kidneys subjected to enzymatic GAG digestion (Kanwar *et al.*, 1980). Glycosaminoglycan related permeability regulation has also been described elsewhere. For example, Parsons showed that across the bladder mucosa, the deletion of GAGs allowed an increase in urea transport across the epithelial layer (Parsons *et al.*, 1990).

### 1.3.5 Structure and function of individual GAG families.

#### 1.3.5.1 Hyaluronic acid

Hyaluronic acid is made up of alternating units of glucuronic acid and N-acetlglucosamine (Figure 5a). It is distinct from other GAGs in that it does not form sulphated bonds and is not bound to a core protein to form a PG. HA is widely distributed in cartilage, the skin, the eye and many tissue fluids. In addition it is found on cell surfaces and as a key part of the ECM. HA receptors are proteins which bind HA and mediate it's effects with the cell. These effects include cell-cell and cell-matrix adhesion and facilitation of cellular migration (Lindahl et al., 1977) (Toole, 1991).

#### 1.3.5.2 Dermatan sulphate

Dermatan sulphate is a connective tissue component found in skin, blood vessels, and heart valves. It's alternative name is Chondroitin sulphate B. The repeating disaccharide unit consists of hexuronic acid and N-acetylgalactosamine (Figure 5.b). Hexuronic acid can be either glucuronic acid or its epimerized form, iduronic acid. Both forms can be found in one individual carbohydrate chain. The disaccharide is often sulphated in position 2 of iduronic acid and position 4 of N-acetylgalactosamine. Its molecular weight is 15-40 kDa and it forms 5-10% of articular cartilage.

### 1.3.5.3 Chondroitin sulphates

Chondroitin sulphates are the most abundant GAGs and are found in cartilage, ligaments, tendons, and the aorta. They constitute up to 80% of the total amount of GAGs in articular cartilage. The constituent disaccharides contain a glucuronic acid and N-acetylgalactosamine (Figure 5c). They are usually but not invariably sulphated. The term Chondroitin sulphate A is used interchangeably with chondroitin-6-sulphate (sulphated on the C6 position of the N-acetylgalactosamine). Chondroitin sulphate C is the alternative name for chondroitin-4-sulphate which is sulphated on the C4 position of the N-acetylgalactosamine.

Enzymatic degradation may be used to determine the differences between CS and DS. Chondroitinase ABC will lyse at glucuronate-containing disaccharides and iduronate-containing disaccharides. Chondroitinase AC will digest only at glucuronate-containing disaccharides (CS), while Chondroitinase B cleaves only at iduronate-containing disaccharides (DS). In experiments for the indirect determination of the tissue distribution of GAG sub-groups, these glycanases were employed. The results are described in Chapter 3 of this thesis.

### 1.3.5.4 Heparan sulphate and Heparin

Heparan sulphate and Heparin have a similar structure consisting of repeating disaccharides of glucoronic acid (or iduronic acid ) and sulphated glucosamine (Gallagher et al., 1986). The size of an individual chain can reach 100 kDa, but normally they are below 50 kDa. Heparin may be considered a sub-class of Heparan sulphate and is the only GAG with predominantly alpha-glycosidic links. Heparin is widely known for its anti-coagulant properties, which are based on its ability to bind with anti-thrombin III (see Figure 5d).

Distinction between H and HS is difficult. They both contain variations of sulphation and L-epimerization. The amount of N-sulphation has occasionally been used to make a distinction between H and HS so that in heparan sulphate the proportion of N-sulphation is below 50%.

Heparan sulphate and Heparin act as regulators of protein function and soluble ligands with a key role in signalling. They also act as co-receptors for other matrix components. In addition they have an important role in the

internalisation of proteins and act as receptors for bacteria. The mechanisms by which they carry out these functions include acting as templates for the assembly of protein complexes, alteration of protein morphology and the binding of proteins such as growth factors, cytokines, enzymes, protease inhibitors and other components of the ECM. With regard to binding, different moieties bind to specific sugar sequences and sulphation patterns confer specific protein binding capabilities.

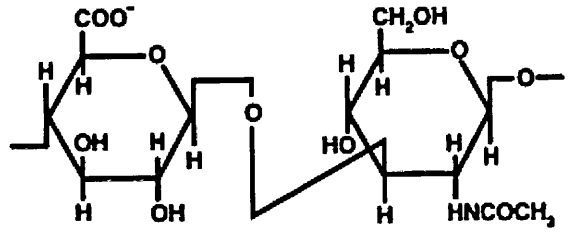
Recent recognition of the importance of H as a trophic agent is important in the context of this thesis. It appears to facilitate epithelial proliferation in a dose-dependent fashion while inhibiting mesenchymal growth (Flint et al., 1994). This may explain some of the consequences of loss of tissue GAGs in inflammatory states.

#### 1.3.5.5 Keratan Sulphate

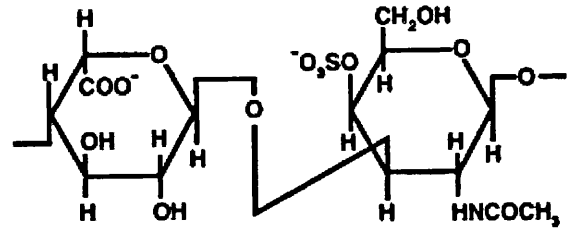
Keratan sulphate is based upon a repeating sequence of galactose and N-acetylglucosamine (Figure 5e). The carbohydrate lengths and the degree of sulphation are variable. The location and main functions of GAGs are summarised in Table 1.1.

**Figure 5** The structure of the basic units of members of the glycosaminoglycan family; (a) Hyaluronic acid is composed of D-glucoronic acid and N-acetlglucosamine with β (1,3) linkage, (b) Dermatan Sulphate comprises L-iduronic acid and N-acetylgalactosamine with β (1,3) linkage, (c) Chondroitin-4-Sulphate and Chondroitin-6-Sulphate consist of D-glucoronic acid and N-acetylgalactosamine with β (1,3) linkage, (d) Heparan Sulphate and Heparin are composed of units of D-glucoronic acid (or iduronic acid ) and sulphated D-glucosamine with α (1, 4) linkage, (e) Keratan Sulphate consists of repeating units of galactose and N-acetlglucosamine with β (1,4) linkage.

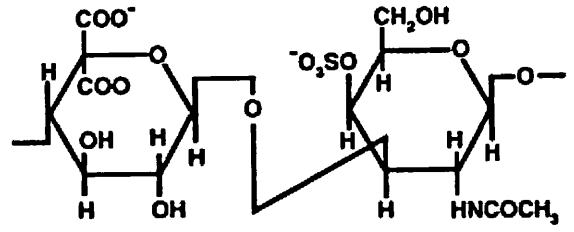
(a)


COO⁻
CH₂OH
HNCOCH₃

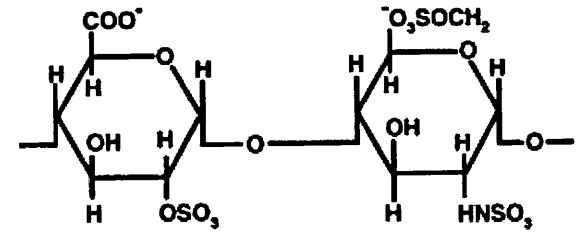
(b)


COO⁻
⁻O₃SO
CH₂OH
HNCOCH₃

(c)


COO⁻
COO
⁻O₃SO
CH₂OH
HNCOCH₃

(d)


COO⁻
⁻O₃SOCH₂
OSO₃
HNSO₃

(e)

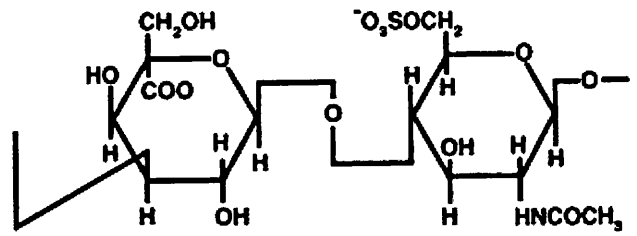

CH₂OH
HO
COO
⁻O₃SOCH₂
HNCOCH₃

**Table 1 Summary of distribution and functions of glycosaminoglycans**

| Glycosaminoglycans | Main locations | Main functions |
|---|---|---|
| Hyaluronic acid. | Cartilage, skin, eye, tissue fluids, cell surfaces, ECM. | shock absorption, cell adhesion, migration |
| Dermatan Sulphate | skin, blood vessels, heart valves, articular cartilage | Structural |
| Chondroitin Sulphate | cartilage, ligaments, tendons, bones, aorta, heart valves | Structural |
| Heparan Sulphate | basement membranes, cell surfaces | regulator protein function, signalling, co-receptor for matrix components, receptor for bacteria |
| Heparin | mast cells, endothelium | regulator protein function, signalling. co-receptor for matrix components, receptor for bacteria, trophic agent |
| Keratan Sulphate | cornea, bone, cartilage | Structural |

ECM, extra-cellular matrix.

## 1.4 Summary and main aims of this thesis

This introduction to NEC does not represent an exhaustive review of the subject but does highlight the fact that in its advanced form, this intestinal disease contributes significantly to morbidity and mortality in the new-born period and chronic ill health in childhood (Kosloske, 1994a).

The clinical presentation of infants with fulminant NEC has been well characterised but little is known about the underlying pathological processes. As a result, the mortality for infants undergoing surgery for NEC has remained unchanged at about 30% over the last two decades (Fasoli *et al.*, 1999; Ade-Ajayi *et al.*, 1996a; Stringer and Spitz, 1993). In the belief that CLS is a key factor determining outcomes in NEC (Sonntag *et al.*, 1998), we sought to understand the mechanism of this phenomenon more clearly.

The complex biology of GAGs and PG's has been simplified in order to emphasise the facts that GAGs are ubiquitous and in addition to their structural role have important homeostatic functions. The main hypothesis of this thesis was that pathophysiological mechanisms in NEC result in

modulation of GAGs. In order to examine this hypothesis, the following main aims were identified:

- To ascertain whether there is evidence of altered GAG tissue distribution in the intestine of infants with NEC.
- To examine the relationship between GAG distribution and the inflammatory cell infiltrate in NEC.
- To determine whether there is *in vivo* evidence of GAG breakdown in an experimental model and human infants.

# Chapter 2

# Materials and general methods

## 2.1 Introduction

Established methods were applied in order to answer the specific questions raised by our hypotheses. In addition, a number of experiments required the adaptation of previously reported techniques. Factors influencing the choice of methodology are described in this chapter. In addition, general methods used are explained and a table of chemicals and reagents is provided. The details of individual experiments are to be found in the relevant chapters and the appendix.

## 2.2 Tissue distribution of glycosaminoglycans

For this series of experiments, a method of GAG detection sufficiently robust for use in preserved neonatal intestine was required. The method also had to be specific for GAGs and avoid the inclusion of other anionic sites such as Sialic acid residues. Furthermore, it was considered important to be able to carry out assessment of stains by light microscopy.

A number of techniques have been developed to assess the distribution of anionic sites in different tissues. Hales Cationic iron, Alcian blue, and Cuprolinic blue have all been used (Chakrabarti et al., 1989). Ruthenium red provides a reliable method but is dependent on electron microscopy (Myers et al., 1973) (Linss et al., 1979). A significant advance came with the report of Skutelsky and Roth on the use of a poly-L-lysine cationic gold probe to detect anionic sites on red blood cell membranes (Skutelsky and Roth, 1986) This method has been validated (Vorbrodt, 1987) and was used by Dikranian in his description of anionic sites in the rat colon (Dikranian et al., 1991). Klein subsequently adapted this method and used it for the detection of GAGs in human umbilical vein (HUVEC). The use of a silver enhancement technique in those experiments heralded the use of light microscopic visualisation with a sensitive method of anionic site

detection (Klein et al., 1993). The method is pH dependent and restriction to a pH of 1.5 is important to avoid the detection of anionic sites such as Sialic acid residues (Klein et al., 1993) (Skutelsky and Roth, 1986).

Having considered the above, we chose poly-L-lysine for the following reasons; reproducibility, ease of use and applicability for light microscopy, ready commercial availability and experience of the use of the method within our unit. A brief description follows.

### 2.2.1 Cationic gold technique

A poly-L-lysine probe (the chain is formed from the amino acid lysine and is highly positively charged), conjugated to 5nm gold particles is applied to tissue sections at a pH of 1.2 -1.5 in a humid environment. Sections are then washed in de-ionised water and developed with a silver enhancer. Meyer's Haematoxylin is used as a counter stain and the slides mounted.

### 2.2.2 Detection of specific GAGs

GAG families were distinguished with the use of Chondroitinase and Heparinase; sections were incubated for 4 hours with Chondroitinase ABC or Heparinase III (Sigma, Dorset, UK) (Klein *et al.*, 1993). Control sections from the same tissue blocks were incubated with phosphate buffered saline (PBS) for 4 hours. Following washing with PBS, the CG stain was then carried out as described above.

### 2.2.3 Examination of the inflammatory infiltrate

Approval of the hospital ethics committee was obtained. Specimens were taken from the proximal margins of intestine resected from infants with NEC. A wide range of disease severity was encountered on examination of this material. Specimens were placed in 10% formalin until fixed (24 - 48 hrs). The tissue was cut into blocks and placed in cassettes. For dehydration purposes and the formation of paraffin blocks, the cassettes were placed in a Shandon Hypercentre 2 for the following programmed cycles: pure formalin, 50% formalin in alcohol, 70% alcohol, 95 % alcohol, pure alcohol (3 cycles), chloroform

and molten wax. The molten wax cassettes were left to solidify. Paraffin sections were cut from an ice base using a Reichert-Jung microtome 2030. Three micron sections were cut from the paraffin blocks and mounted on Vectabond coated slides (Vector laboratories SP1800). The sections were dewaxed and in addition sections for CD68, CD3 and CD20 immunohistochemistry were subjected to high temperature antigen unmasking (Cattoretti *et al.*, 1993).

In order to dewax, the slides were immersed in Histoclear I for 10 minutes, then Histoclear II for another 10 minutes. They were then put through decreasing alcohol concentrations starting from pure alcohol (two runs of 3 minutes). From 30% alcohol, the slides were placed in phosphate buffered saline (Dalbecco) and then rinsed with water.

Sections from each patient were stained with Meyer's haematoxylin, washed in running tap water, differentiated in 1% acid alcohol, washed again in running water and counterstained in 1% eosin.

### 2.2.4 Immunohistochemistry

Recent advances in immunohistochemistry have made the detailed characterisation of the ICI possible. For example, polyclonal anti-elastase antisera raised against human neutrophil elastase (Crocker et al., 1984), (Kramps et al., 1984) has been characterised by affinity chromatographic separation of the neutrophil granule proteins (Pulford et al., 1988). The epitope, NP57, detected by neutrophil elastase is formalin resistant, making it a suitable antibody for use in paraffin sections. Some of the antigens specific for components of the ICI are robust, others are more subtle and require unmasking (Cattoretti et al., 1993).

#### 2.2.4.1 Available methods

A large number of indirect systems are available for immunohistochemical antigen localisation (Belling et al., 1999). Many of these are novel techniques and have not been widely validated. Methods in more general use, with proven efficacy, include those based on enzyme anti-enzyme complex reactions such as the Alkaline Phosphatase Anti-Alkaline

Phosphatase (APAAP) technique and those that depend on avidin-biotin interactions (Mokry, 1996).

### 2.2.4.2 Choice of method for the study

The primary factors considered in arriving at a choice were sensitivity, specificity and clarity of staining. Other considerations were cost, ease of use and time consumption. The APAAP technique is a sensitive and relatively easy method to perform and is applicable in paraffin-embedded sections (Kurec et al., 1988). It has the disadvantage of being more expensive than other systems (Belling et al., 1999), but provides excellent antigen definition and is therefore widely used (Davey et al., 1990, Lelle, 1990). For the majority of immunohistochemical experiments, the APAAP technique was employed with a panel of monoclonal antibodies to detect components of the inflammatory infiltrate.

### 2.2.4.3 The APAAP technique

Primary mouse monoclonal antibody was applied and incubated for 30 minutes. Rabbit anti-mouse immunoglobulin and APAAP mouse

monoclonal complex were each incubated for 30 minutes with intervening 5 minute washes using Tris buffered saline (TBS). A fast red alkaline phosphatase substrate was then applied for 20 minutes and the reaction terminated in water. Meyer's Haematoxylin was used as a counterstain and the slides mounted.

The expression of adhesion molecules was also determined using the APAAP technique following high temperature citration. Goat polyclonal antibody was applied to the slides and was followed by incubation with the appropriate monoclonal antibody IgG. Fast red and Meyer's haematoxylin were applied as previously described.

#### 2.2.4.4 The Extravidin Biotin Peroxidase technique

In a minority of experiments, in order to validate findings related to CD3 and CD20, the Extravidin Biotin Peroxidase technique was employed for antigen detection. Endogenous peroxidase was blocked with 10% hydrogen peroxidase in PBS. Following high temperature antigen unmasking, slides were incubated with primary antibody for 1 hour and then secondary antibody for a further hour. Incubation in Extravidin for

another hour was then followed by development of peroxidase activity over 10 minutes and counterstaining with Meyer's haematoxylin.

## 2.3 Urinary Glycosaminoglycan determination

### 2.3.1 Available methods

The development of assays for the urinary estimation of GAGs has primarily been driven by the need to screen for mucopolysaccharidoses (Piraud *et al.*, 1993; Dembure *et al.*, 1990). Spot and turbidity tests are inaccurate and older quantification techniques (Di Ferrante, 1967), time consuming. The Alcian-Blue method reported by Whiteman in 1973 (Whiteman, 1973a; Whiteman, 1973b), simplified the methodology for quantitative GAG detection considerably and for many years was the gold standard. More recently assays based on adaptations of the technique, which have the advantages of further simplicity and exclusion of urinary protein (de Jong *et al.*, 1989), have gained wide acceptance.

For basic qualitative analysis of GAGs in urine, one or two dimensional electrophoresis followed by an appropriate stain may be used. Two dimensional electrophoresis is more time consuming but has the advantage of better discrimination between GAG sub-groups. Solvents for separation

include barium acetate, calcium acetate, barbital buffer and pyridine-formic (Kodama et al., 1988). In order to achieve complete separation of all GAG sub-groups, more complex methods such as discontinuous electrophoresis are required.

### 2.3.2 Choice of method for the study

Whiteman's original observations on quantification were made from this department. Given years of experience with it's application (and more recently with the de Jong modification of the technique (de Jong *et al.*, 1989), these were the logical choices for this project. For electrophoresis, a two dimensional technique on Sartorius cellulose acetate sheets was employed. Descriptions follow.

#### 2.3.2.1 Quantitative GAG estimation

Under controlled pH and electrolyte conditions, Alcian blue added to urine reacts with GAGs, forming insoluble complexes. For these experiments, the complexing agent was 0.05% Alcian Blue in 5 mmol/L magnesium chloride, with 500 mmol/L of sodium acetate buffer at pH 5.8. This was prepared fresh and spun down, giving a clear blue supernatant. Urine samples were also spun down and 50 µl samples of urine and standard added to 1ml of the complexing reagent in microfuge tubes and allowed to stand for 2 hours. Centrifugation was then carried out at 10,000 rpm (Biofuge Pico Heraens), following which the supernatant was discarded

and the precipitate washed twice in absolute ethanol. Next, the Alcian blue - GAG complex was dissolved in 1 ml of 7.5% sodium dodecyl sulphate prior to measurement by spectrophotometry. A standard curve was set up using chondroitin-4-sulphate and absorbance was read at 678nm on a spectrophotometer (Cobas Bio 22-3694).

#### 2.3.2.2 Qualitative GAG analysis

For qualitative analysis, a cellulose acetate sheet was soaked in pyridine/acetic acid buffer, blotted and placed in a tank with the application point 1 cm from the cathode. Wicks were placed over the paper edges and samples applied and allowed to dry.

Electrophoresis was run for 1.5 hours in pyridine/acetic acid with an applied potential of 7.5 v/cm. (constant voltage – 50 V) in the direction of the anode. The sheets were then removed, dried and soaked in 0.1mmol/Lbarium acetate and blotted. The sheets were placed in a barium acetate tank at 90 degrees and electrophoresis carried out for a further 3.5 hours in barium acetate with applied potential 7.5 v/cm (constant voltage – 50 V) in the direction of the anode. The sheets were removed and stained with Alcian blue.

## 2.4 Chemicals and reagents

**Table 2 Chemicals and reagents**

| Chemicals and reagents | Source | Address |
|---|---|---|
| Acetic acid | BDH | Poole, UK |
| Alcian blue 8GX | ICI | Manchester, UK |
| APAAP mouse monclonal | DAKO | High Wycombe, UK |
| Aquamount | BDH | Poole, UK |
| Barium acetate | BDH | Poole, UK |
| Cellulose acetate | Sartorius | Gottigen, Germany |
| Anti-human T cell | DAKO | High Wycombe, UK |
| Anti-human B cell | DAKO | High Wycombe, UK |
| Anti-human ICAM-1 | R&D systems | Abingdon, UK |
| Anti-humanVCAM-1 | R&D systems | Abingdon, UK |
| Anti-human E-Selectin | R&D systems | Abingdon, UK |
| Chondroitinase ABC | Sigma | London, UK |
| Chondroitinase AC | Sigma | London, UK |
| Chondroitin - 4 - Sulphate | Sigma | London, UK |
| Chondroitin - 6 - Sulphate | Sigma | London, UK |
| Citric acid | Sigma | London, UK |
| DPX | BDH | Poole, UK |

| | | |
|---|---|---|
| Formaldehyde | BDH | Poole, UK |
| Gold conjugated poly-l-lysine | Biocell | Cardiff, UK |
| Heparinase III | Sigma | London, UK |
| Anti-human HLA-DR | DAKO | High Wycombe, UK |
| Anti-human Ki-67 | DAKO | High Wycombe, UK |
| Anti-human myeloid | DAKO | High Wycombe, UK |
| Magnesium chloride | Sigma | London, UK |
| Methanol | BDH | Poole, UK |
| Anti-human neutrophil elastase | DAKO | High Wycombe, UK |
| Paraformaldehyde | BDH | Poole, UK |
| Rabbit anti-mouse Ig | DAKO | High Wycombe, UK |
| Silver enhancer | Biocell | Cardiff, UK |

# Chapter 3

# Distribution of intestinal glycosaminoglycans in neonatal necrotizing enterocolitis

## 3.1 Introduction

The focus of histopathological research into NEC has been the impact of the disease on intestinal architecture. The cellular infiltrate has been examined to a lesser degree and very little attention has been paid to the role and fate of components of the ECM. The development of means to identify and quantify components of the ECM has resulted in an increasing interest in their role in general and in intestinal pathophysiology in particular (Vantrappen and Geboes, 1993).

### 3.1.1 GAG distribution and functions in the intestinal tract

The sulphated GAGs heparan, dermatan and chondroitin, are widely distributed in normal intestine (Iozzo and Wight, 1982) (Dikranian et al., 1991) and their modulation may underlie many of the pathological features of gut inflammation. Experiments carried out on human umbilical vein endothelial cells (HUVEC), suggest that alterations in GAG metabolism induced by inflammatory mediators may explain loss of albumin into the interstitial compartment with subsequent capillary leak (Klein et al., 1992). The beneficial effects of H in intestinal inflammation are mediated by anti-ulcerogenic moieties, such as fibroblast growth factor which depend on

proteoglycans such as syndecan-1 as co-receptors. Degradation of these proteoglycans may result in an inability to bind fibroblast growth factor (FGF) and impaired healing of mucosal ulceration (Day and Forbes, 1999) (Day et al., 1999).

### 3.1.2 Intestinal distribution of GAG sub-groups

Of the sub-groups of GAGs, HS and CS are the best studied in the intestine. Indirect immunofluorescence has established that HS proteoglycan, along with other ECM components, is present at the epithelial-mesenchymal interface of human foetal small intestine from 8 weeks of gestation (Beaulieu et al., 1991). The pattern of distribution of ECM components in experimental animals and humans, when correlated with the morphological development of the intestine, suggests they have an important role in early intestinal remodelling (Simon-Assmann et al., 1989) (Beaulieu et al., 1991). For example, in the developing rat intestine anti-HS proteoglycan antibody demonstrated strong labelling in the membrane zone on day 14 prior to villous morphogenesis. By day 18, basement membrane labelling was continuous along the villi and in peripheral mesenchyme. With further development, this stain along the

villi became discontinuous, especially at the apices of the villi. Immunolocalisation of HS proteoglycan in adult rat intestine demonstrated a distribution that was most prominent in the basement membrane lining the epithelium and along the crypt-villous axis in the lamina propria and longitudinal circular muscle layers (Simon-Assmann et al., 1989). Furthermore, it has been established that epithelial cells are the cellular source of HS proteoglycans. Despite these observations, there is much about the morphology and the role of intestinal GAGs, in health and in intestinal disease, which is poorly understood.

### 3.1.3 Glycosaminoglycans in intestinal disease

In a study of 17 patients with inflammatory bowel disease and 8 controls, Murch e al demonstrated the extensive presence of GAGs in basal lamina and in the extra-cellualr matrix of the submucosa in normal adult bowel. The distribution of sulphated GAGs in Ulcerative Colitis and Crohns disease was described, demonstrating mucosal disruption in the former and submucosal loss in the latter. They concluded that the vascular and connective tissue GAG disruption seen in inflammation may be important in protein and fluid leakage from the gastrointestinal tract (Murch et al., 1993).

In the light of these findings, similar mechanisms were considered in infants with NEC. In particular, loss of the barrier function of negatively charged GAGs resulting in intestinal protein loss, hypo-albuminaemia and the capillary leak syndrome (CLS) was proposed as a possible mechanism. We hypothesised that GAGs in the neonatal GI tract are disrupted in NEC in proportion to the severity of the disease. We examined the relationship between intestinal GAG distribution and severity of NEC as a step towards determination of the role of GAGs in the patho-physiology of this condition. In this chapter, experiments to determine the intestinal GAG distribution in NEC are described using techniques outlined in Chapter 2. Established glycanases were used to determine predominant distribution sites of specific members of the GAG family.

## 3.2 Methods

### 3.2.1 Patients and samples

Thirty-one intestinal tissue sections from 8 patients who underwent surgery for NEC were studied in this series of experiments. Hospital ethical approval was obtained. The median gestational age was 33.5 weeks (range 27-38 weeks) and median birth weight 1593g (range 705-2908g). Age at the time of surgery ranged from 2 to 62 days (Table 3). Samples were placed in 10% formalin post-operatively, embedded in paraffin and 3 micron sections prepared. The sections were stained and examined. Areas of severe NEC were compared with well preserved resection margins that were used as controls.

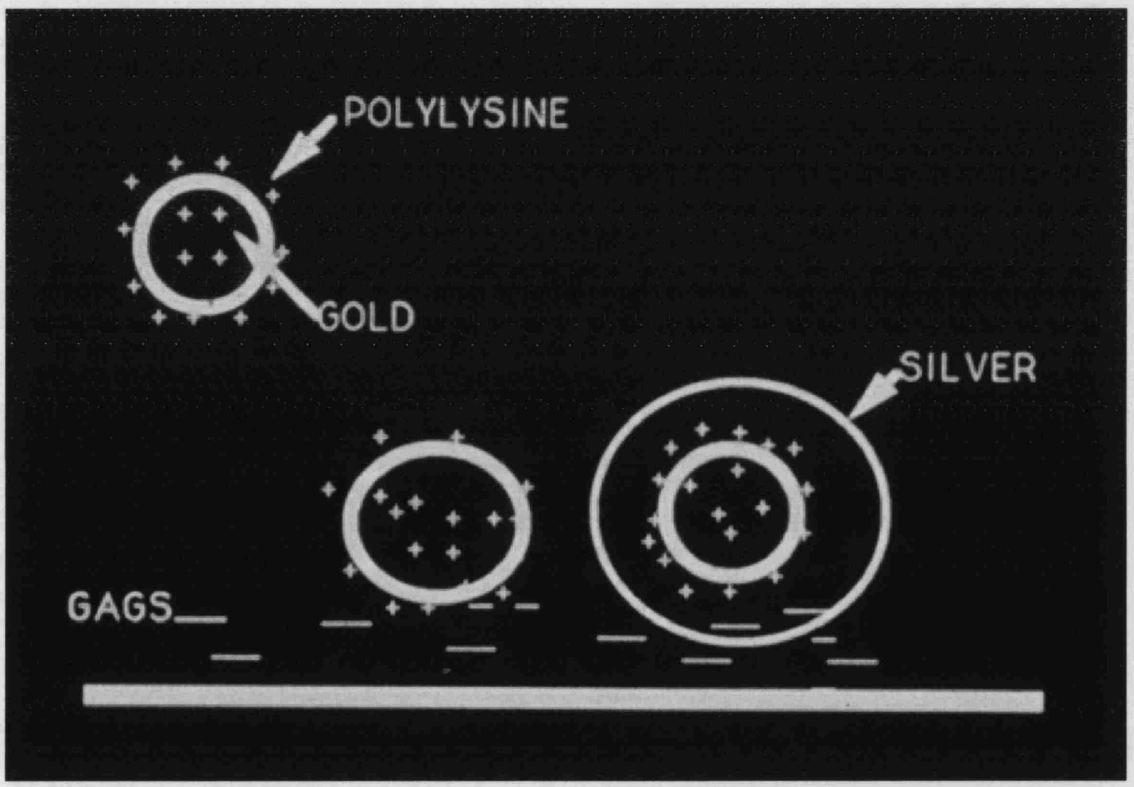
**Table 3 Infants who underwent bowel resection for NEC and determination of tissue GAGs**

| No | Sex | Gestational age | Birth Weight (g) | Age at surgery(days) |
|---|---|---|---|---|
| 1 | M | 33 | 1660 | 13 |
| 2 | F | 27 | 843 | 18 |
| 3 | M | 38 | 2980 | 3 |
| 4 | F | 28 | 705 | 20 |
| 5 | M | 37 | 2300 | 9 |
| 6 | M | 30 | 1526 | 12 |
| 7 | M | 37 | 2125 | 2 |
| 8 | F | 34 | 1332 | 62 |

### 3.2.2 Distribution of GAGs

Specimens taken at laparotomy were fixed, placed in paraffin blocks then dewaxed as described in Chapter 2. GAGs were detected using a cationic gold (CG) method as previously described (Klein *et al.*, 1993). Briefly, lysine constituted into a chain of repeating sequences of the amino acid forms a highly positively charged probe poly-L-lysine. Conjugated to 5nm gold particles (Biocell Research laboratories, Cardiff, UK) the probe was applied to each slide for 60 minutes at a pH of 1.2 -1.5 in a humid environment. Each slide was then washed in de-ionised water and developed with a silver enhancer (Figure 6). Meyer's Haematoxylin was used as a counter stain for 1 minute and Aquamount (BDH, Essex England) was used for slide mounting. The slides were allowed to dry and then viewed under a light microscope (Olympus BH-2). Sections from the same blocks were also stained with haematoxylin and eosin (H&E) in order to make direct comparisons.

**Figure 6** The colloidal gold method for the detection of glycosaminoglycans.




*Klein 1993*

### 3.2.3 Characterisation of GAGs

Histological examination was carried out using an Olympus BH-2 light microscope. Modification of an existing histological score for assessing disease severity on standard H&E sections of experimentally induced NEC in neonatal piglets was carried out. Sibbons scored parameters including vessel engorgement, mucosal ulceration, sloughing, pneumatosis and necrosis on a scale of 1-5 (Sibbons *et al.*, 1988). For the purpose of this study, this scoring system was modified by considering each bowel section in 4 layers; the epithelium, lamina propria and muscularis mucosa, submucosa and serosa. To reduce inter and intra-observer variation, the number of parameters in each layer were reduced from 5 to 4. In sections stained with H&E, a score of 0 was given for normal appearance, 1 for mild, 2 for moderate and 3 for severe changes in each layer. On sections stained with CG, corresponding layers were examined and similarly scored on a scale of 0-3 to quantify the presence and intensity of anionic staining. A score of 0 represented intact distribution and normal intensity and 3 an absence of anionic sites. The same criteria were used to analyse sections stained following enzymatic digestion of GAGs. The maximum scores indicating the most severe disease with both H&E and CG staining was 12.

To elucidate the nature of GAGs in neonatal bowel, specific glycanases were employed. The material used for this batch of experiments came from infants in whom the resection margins had a histological severity score of $\leq 4$. Sections were incubated for 4 hours with Chondroitinase ABC or Heparinase III (Sigma, Dorset, UK) (Klein *et al.*, 1993). Control sections from the same tissue blocks were incubated with phosphate buffered saline (PBS) for 4 hours. Following washing with PBS, the CG stain was then carried out as described above.

## 3.3 Results

### 3.3.1 GAG distribution in well preserved neonatal bowel

On the basis of H&E staining, the 31 sections were divided into severe, moderate and well-preserved categories (Table 4). Five sections from 3 of the neonates examined demonstrated well preserved architecture, few inflammatory cells and little evidence of vessel dilatation or haemorrhage on H&E staining (score $\leq 4$). In this series of experiments, these sections were used as controls and were analysed for the distribution of anionic sites as described above. The results were taken as an approximation of the situation in healthy neonatal bowel.

All layers of well preserved intestine were stained (Table 5.). In the epithelial layer, staining was most prominent in the basement membrane and baso-lateral surfaces (Figure 7). Goblet cells stained frequently and there was CG staining in the lamina propria with a network of fine interlacing strands.

In the sub-mucosa the staining pattern was intense and predominantly associated with the collagen matrix (Figure 8). Medium and large sized vessels were also heavily stained with CG deposited on the endothelial basement membrane, smooth muscle cells and peri-vascular region.

**Table 4 The trend towards increased GAG degradation in severe NEC**

| Grouped severity score (H&E) | H&E Severity Score (mean +/- SD) | CG Score (mean +/- SD) |
|---|---|---|
| Severe; score 9-12 (n=17) | 11.4 +/-0.86 | 8.8 +/- 1.59 |
| Moderate; score 5-8 (n=9) | 6.7 +/- 1.3 | 7.3 +/- 2.6 |
| Well preserved; score 1-4 (n=5) | 3 +/- 1.4 | 4.4 +/- 1.5 |

SD, standard deviation; H&E, haematoxylin and eosin; CG, cationic gold.

**Table 5 Distribution of anionic sites in neonatal intestine affected by NEC**

The findings are those on light microscopy following the use of a cationic gold stain at a pH of 1.5. Well preserved and severely affected sections are compared.

| Site | Severity score ≤ 4 | Severity score 9-12 |
|---|---|---|
| Epithelial surface covering | ++ | + |
| Nucleus | ++ | ++ |
| Baso-lateral surface | +++ | - |
| Epithelial Basement Membrane | +++ | - |
| Lamina Propria | +++ | + |
| Submucosa | +++ | + |
| Smooth Muscle Cells | +++ | ++ |

- absent

+ occasionally present/severely attenuated

++ commonly present/moderately attenuated

+++ consistently present/intact

**Figure 7** Photomicrograph of intestinal resection margin of a patient affected by necrotizing enterocolitis showing colloidal gold (brown stain) deposited in the basement membrane, baso-lateral epithelial surfaces and lamina propria (CG stain - original magnification x 40).

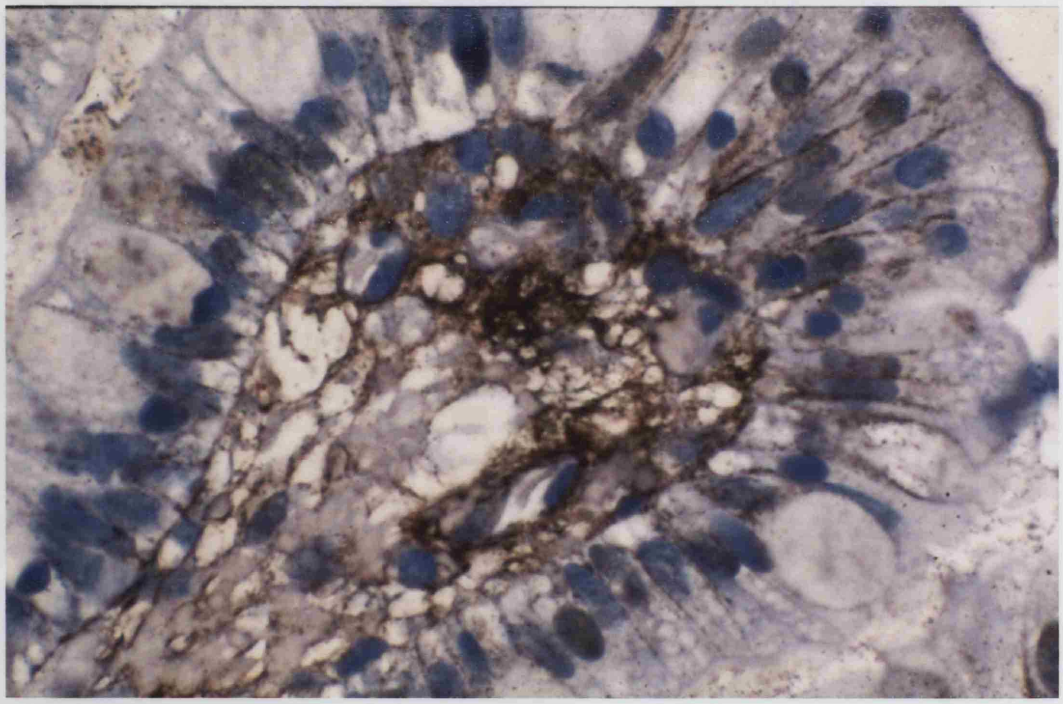
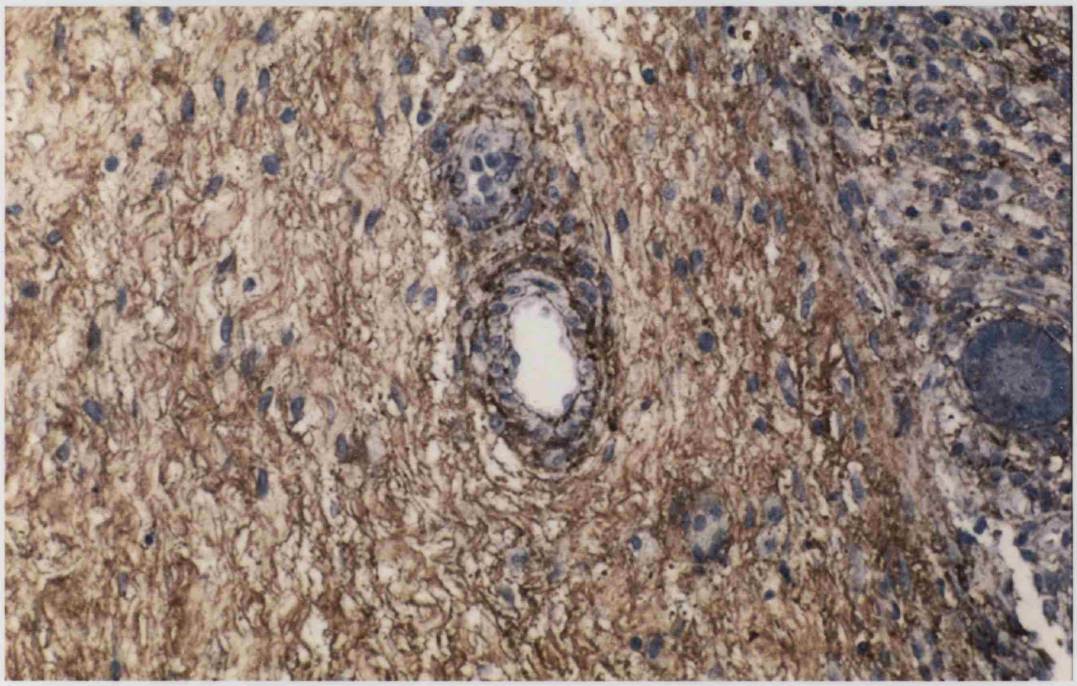


**Figure 8** Well preserved resection margin with intense peri-vascular and submucosal matrix glycosaminoglycans (original magnification x 10).



### 3.3.2 GAG distribution in diseased bowel

Sections with histological evidence of severe NEC, stained with H&E (n = 17) were characterised by mucosal ulceration and stripping, a heavy inflammatory infiltrate and coagulative necrosis. These were scored at between 9 and 12 (Table 4.). Generally, NEC severity was mirrored by an increase in GAG loss (Table 5). The baso-lateral and basement membrane GAGs in these sections, were attenuated in all samples. Submucosal matrix GAGs were affected in the most severe cases.

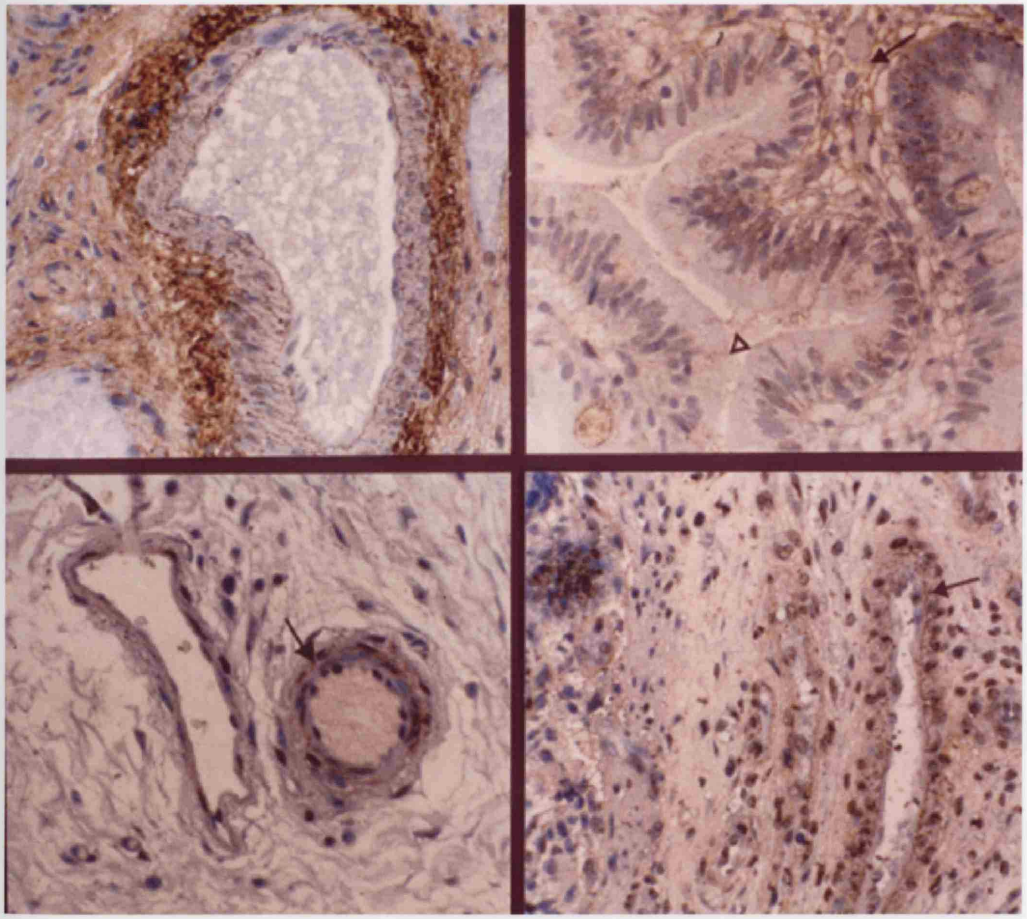
Compared with well-preserved tissue (Figure 9a), sections with moderate evidence of disease on H&E staining had a variable GAG stain, with deficient areas often adjacent to intact sites (Figure 9b). Severe histological disease was characterised by profound GAG degradation (Figure 9c).

### 3.3.3 Nature of GAGs in neonatal bowel

When Chondroitinase ABC was applied to well preserved bowel (n = 5), GAGs in all layers of the bowel wall except the sub-mucosa were digested. The submucosal peri-vascular stain was the most resilient to this particular glycanase (Figure 9d). The similarities between the Chondroitinase digested bowel and the appearance of sub-mucosal loss in severe disease (Figure 9c) are apparent. Whilst vascular GAGs were reduced with Heparinase III, basement membrane and baso-lateral GAGs were largely unaffected.

**Figure 9** Spectrum of colloidal gold (CG) stain in necrotizing enterocolitis (NEC).

(a) A large submucosal vessel in well preserved neonatal colon with intense peri-vascular CG stain (original magnification x 40).
(b) Moderate NEC. Arrow shows well preserved glycosaminoglycans (GAGs) adjacent to areas of attenuation (arrowhead) (CG stain - original magnification x 40). (c) Severe NEC with widespread disruption of sub-mucosal GAGs but limited preservation of vascular GAGs (arrow) (original magnification x 40). (d) Neonatal colon following incubation with Chondroitinase ABC showing preservation of vascular GAGs (arrow) and digestion of matrix anionic sites (CG stain - original magnification x 40).



## 3.4 Discussion

There are few detailed reports of the nature and distribution of GAGs in paediatric and adult bowel, and no published descriptions of GAGs in human neonatal intestine. A major reason for this is the difficulty in obtaining normal gastrointestinal tissue in neonates. We have selected healthy resection margins as an approximation of normality. Using the CG probe technique described for electron microscopy by Skutelsky in 1986 (Skutelsky and Roth, 1986), modified for light microscopy (Klein et al., 1993), we have examined neonatal bowel from babies with NEC.

In well-preserved tissue, GAGs were distributed in all layers of the small and large neonatal intestine. In common with older children and adults (Murch et al., 1993), baso-lateral, epithelial surface, basement membrane, sub-mucosal and serosal GAGs were particularly prominent. These findings are also similar to those described by Dikranian in a CG study of normal rat colon (Dikranian et al., 1991). In contrast to the pattern seen in well preserved tissue, CG deposition was markedly reduced in all layers in severe disease. In this process, mucosal loss occurred earlier than sub-mucosal and serosal GAG attenuation. In sections with moderate histological disease, epithelial and lamina propria GAG distribution was

frequently patchy with areas of attenuation adjacent to well preserved CG sites. This is consistent with the patchy nature of NEC on H&E sections (Kliegman et al., 1993). The overall distribution patterns were consistent between small and large intestine. Cationic gold staining of the basement membrane, in both parts of the bowel were affected similarly by Heparinase III and Chondroitinase ABC, suggesting that vascular anionic sites were mainly HS while those in the basement membrane, baso-lateral surfaces and submucosal matrix were predominantly CS.

GAGs have an important role in gastrointestinal development. In animal studies of epithelial - mesenchymal interactions, HS was shown to be essential for regulating cell behaviour during morphogenesis (Simon-Assmann et al., 1989). A variety of mechanisms influence GAG distribution and metabolism. Inflammatory cytokines, proteases and glycanases, released from inflammatory cells have all been shown to attenuate tissue GAGs (Klein et al., 1992). These mechanisms are thought to be responsible for the loss of GAGs seen in inflammatory bowel disease. Murch demonstrated that in ulcerative colitis and Crohns disease, the severity of inflammation was accompanied by a loss of GAG staining (Murch et al., 1993) and was most marked in areas of inflammatory cell infiltration. Similar mechanisms were thought to operate in NEC.

It is clear from this study that GAG attenuation does occur in NEC. The underlying cause of the GAG loss observed is not known. Whether infants with NEC suffer direct patho-physiological consequences as a result of GAG degradation also requires further investigation. In other systems, the result of such loss has been demonstrated. For example, Kanwar reported that experimental removal of HS from the glomerular basement membrane by digestion resulted in dramatic increases in leakage of large macro-molecules (Kanwar et al., 1980). In the light of the data presented here and such previous studies of GAG attenuation in other systems, we speculate that GAG loss from vascular and epithelial surfaces may aggravate albumin and fluid leakage and contribute to the development of the CLS. Furthermore, alterations in GAG functions such as structural integrity, cellular traffic, growth factor binding and thrombo-resistance may contribute to the patho-physiology of NEC. Further investigations into the role of GAGs and other extra-cellular matrix components in NEC may provide clues to the aetiological and patho-physiological mechanisms that underlie this condition.

## 3.5 Conclusions

The results reported in this chapter confirm that gold conjugated poly-L-lysine is a useful tool for the visualisation of intestinal anionic sites in neonatal intestine. The technique can reliably be applied to paraffin embedded tissue sections, making it available for the investigation of preserved intestinal tissue from infants who have undergone resectional surgery for NEC.

Vascular, matrix and cell surface loss of GAGs was proportionate to histopathological severity of NEC as scored on H&E stained sections. In addition, indirect evidence of GAG patterns in the bowel wall suggest that vascular GAGs were mainly HS while in the basement membrane, baso-lateral surfaces and submucosal matrix CS predominated.

The first question that arises from these experiments is whether the observed GAG degradation is merely a secondary phenomenon such as a non-specific response to tissue ischaemia. The data demonstrating a graded degradation with increasing NEC severity suggests otherwise. We

therefore hypothesised that GAG loss occurs in response to the presence of glycanases released as a result of NEC. Cells that produce glycanases include neutrophils, platelets, mast cells and macrophages. We undertook a further series of experiments to answer this question. The experiments are described and the results reported in the following chapter.

# Chapter 4

# Glycosaminoglycan distribution in relation to neutrophils and macrophages.

## 4.1 Introduction

Having demonstrated GAG degradation associated with NEC in the work presented in the preceding chapter, the key question raised was whether the observations simply reflect end stage events secondary to intestinal ischaemia. The clear relationship between increasing histological disease severity on H&E sections and graded loss of GAGs suggests that this was not the case and raises questions about the mechanisms underlying the attenuation of GAGs in NEC.

### 4.1.1 Mechanisms of GAG attenuation

Depolymerisation of GAGs may occur as a result of inflammatory cell infiltration. Using column chromatography and comparing free radical fluxes between a neutrophil system and cell-free superoxide system, Greenwald demonstrated physical disruption of the HA macromolecule in response to free oxygen radical production (Greenwald and Moak, 1986). They concluded that neutrophils operating via oxygen-derived free radicals are probably the major mechanism by which HA is degraded.

Neutrophils, platelets, macrophages, lymphocytes and mast cells all secrete a variety of glycanases (Pender et al., 1996, Moseley et al., 1997). We hypothesised that components of the intestinal inflammatory cell infiltrate have a direct effect on the process of GAG degradation in NEC. This chapter describes work designed to answer the specific question of whether or not a direct spatial relationship exists between the cellular infiltrate in NEC and localised GAG degradation.

## 4.2 Methods

A double staining immunohistochemical technique was applied to archival small bowel from infants with NEC.

### 4.2.1 Patients

Twenty neonates who underwent intestinal resection and a primary anastomosis in which the proximal resection involved small intestine were studied. The median gestational age was 28 weeks and birth weight 1220g. At the time of surgery, the median age of the infants was 15 days. Samples were obtained at laparotomy and handled as described below.

### 4.2.2 Tissue preparation

For the purposes of standardisation, all specimens examined in this series of experiments were taken from the proximal resection margin. The tissue was placed in 10% formalin and embedded in paraffin. Three micron sections were cut from the paraffin blocks and mounted on Vectabond coated slides (Vector laboratories SP1800). The sections were dewaxed and, in addition, sections for CD68 immunohistochemistry were subjected to high temperature antigen unmasking (Cattoretti *et al.*, 1993).

### 4.2.3 Haematoxylin and eosin

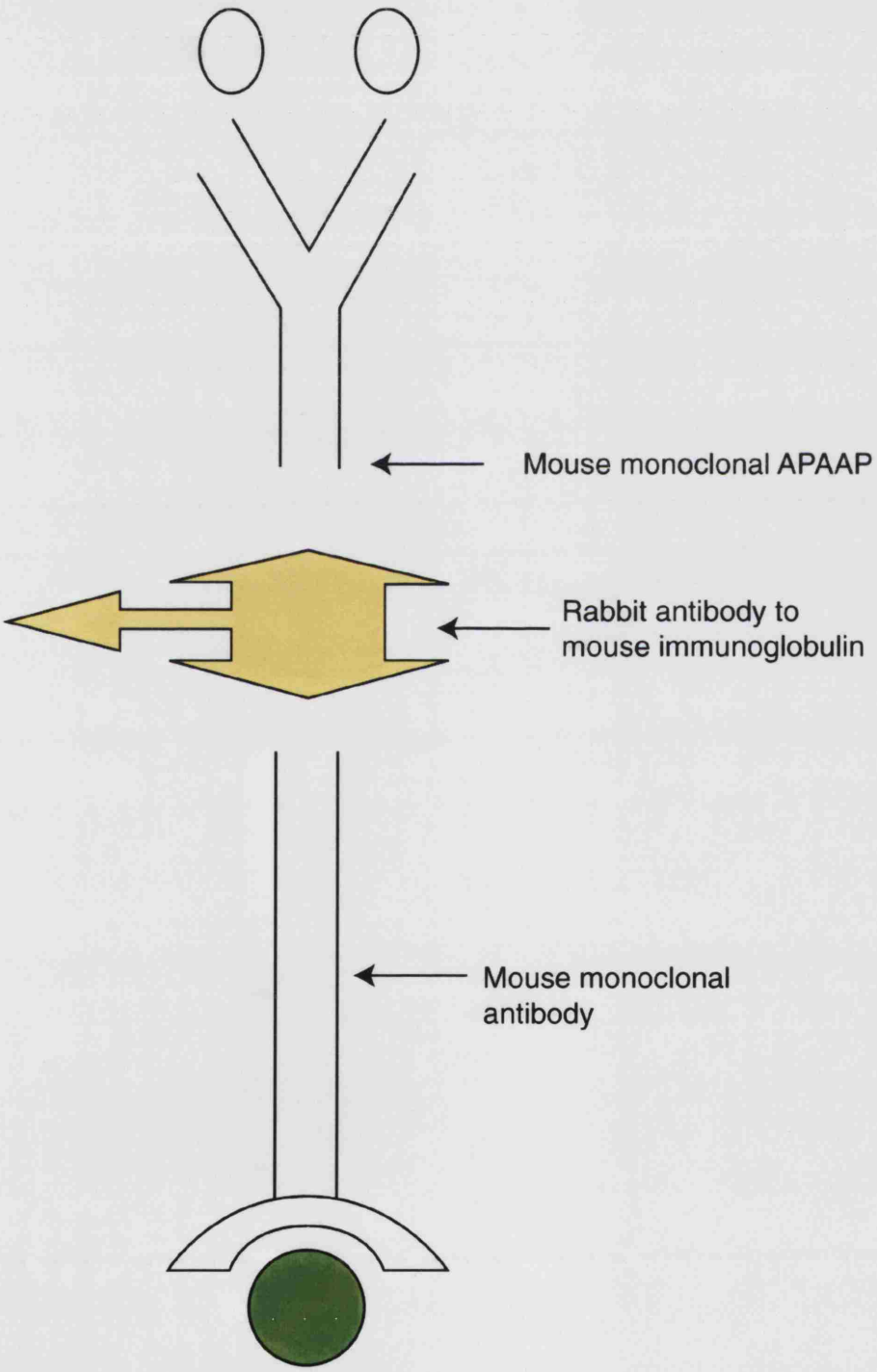
Sections from each patient were stained with Meyer's haematoxylin, washed in running tap water, differentiated in 1% acid alcohol, washed again in running water and counterstained in 1% eosin.

### 4.2.4 Cationic gold histochemistry and APAAP technique

A double staining technique was utilised for the simultaneous detection of GAGs and inflammatory cell subgroups. The CG gold technique was employed as described in chapter 3.

Employing the APAAP technique (Figure 10), the monoclonal antibodies neutrophil elastase and MAC 387 were used to identify neutrophils and cells of the monocyte macrophage line. Primary mouse monoclonal antibody was applied (dilution 1:50) and incubated for 30 minutes. Rabbit anti-mouse immunoglobulin (1:25 dilution - DAKO Code no. Z 0259) and APAAP mouse monoclonal complex at a dilution of 1:50 (DAKO Code no. D0651) were each incubated for 30 minutes with intervening 5 minute washes using TBS in between. A fast red alkaline phosphatase substrate was then applied for 20 minutes and the reaction terminated in water. Meyer's Haematoxylin was used as a counterstain and the slides mounted in Aquamount (BDH, Essex, UK).

**Figure 10** The alkaline phosphatase anti-alkaline phosphatase (APAAP) bridge technique for antigen localisation. The green ball represents the antigen.



### 4.2.5 Histological examination

An Olympus BH-2 light microscope was used. Overall assessment of each section at low and high power magnification was carried out for descriptive purposes. In addition, high power fields of epithelium, lamina propria and muscularis mucosa, submucosa and serosa were analysed as described in chapter 3. Briefly, in sections stained with H&E, each layer was scored for disease severity using haemorrhage, ulceration, architectural destruction and necrosis as parameters. Sections were classed as being well preserved, moderately diseased and severely diseased based on the overall score. The degree of GAG degradation in each layer was then related to the intensity of the cellular infiltrate. Particular attention was paid to the submucosal vascular and peri-vascular cellular infiltrate in relation to GAG intensity.

## 4.3 Results

### 4.3.1 Evidence of GAG attenuation in NEC

Re-assessment of GAG distribution in NEC in this additional group of patients confirmed the findings described in Chapter 3. In well-preserved sections, GAG staining was evident in all layers of the bowel wall. There were few acute inflammatory cells evident at this stage of histological severity as demonstrated by the CG and NE double stained section (Figure 11). Attenuation of GAGs with increasing NEC severity scores was also confirmed in all layers of the intestinal wall. GAG loss invariably occurred in the mucosa and lamina propria before the submucosa and serosa were affected.

### 4.3.2 Glycosaminoglycan degradation with leucocyte adherence to vascular endothelium

With regard to their relationship to GAGs, neutrophils and cells of the monocyte macrophage line were similar. Adherence of both cell types to

vascular endothelium was often demonstrated. Localised breakdown in the continuity of GAGs in relation to this was a frequent though not a universal finding. Figure 12. demonstrates neutrophil adherence to the endothelial layer of a post capillary venule.

**Figure 11** Colloidal gold and neutrophil elastase double staining in mild necrotizing enterocolitis showing glycosaminoglycans (brown stain) in all layers of the bowel wall. Only a few neutrophils (arrows) are seen at this stage (original magnification x 5).

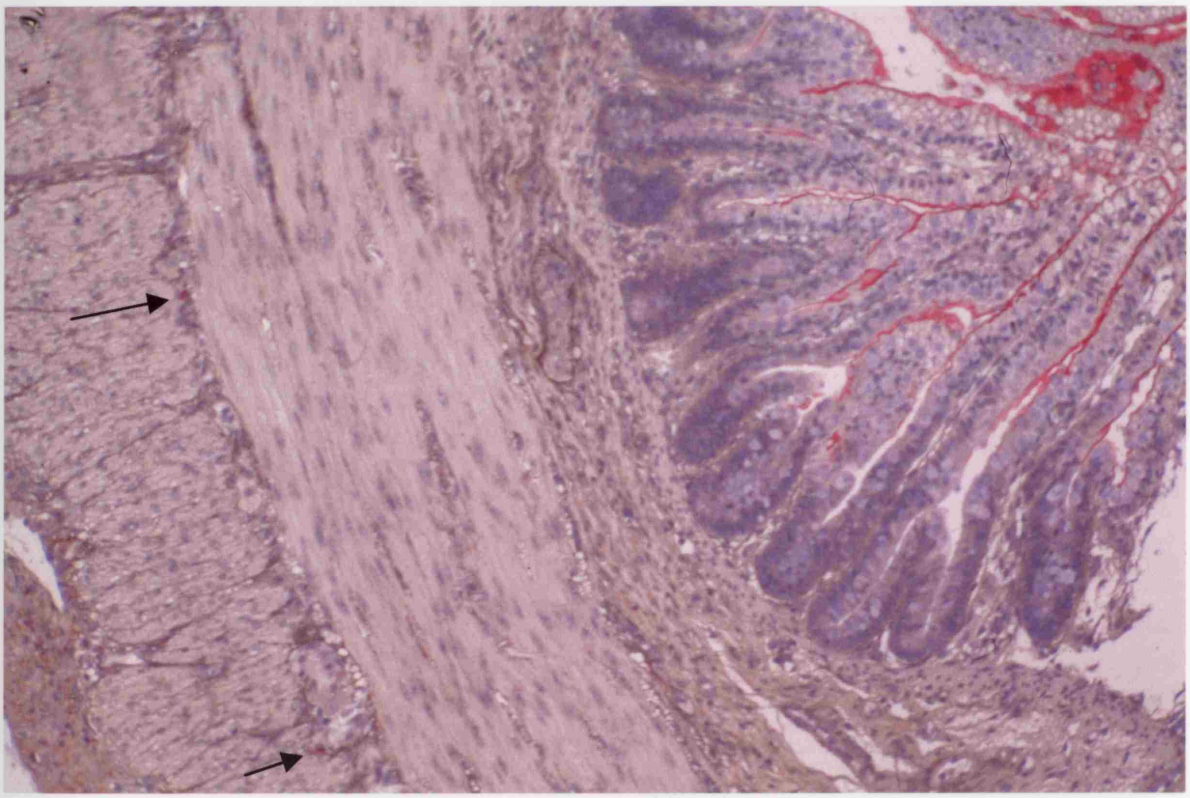
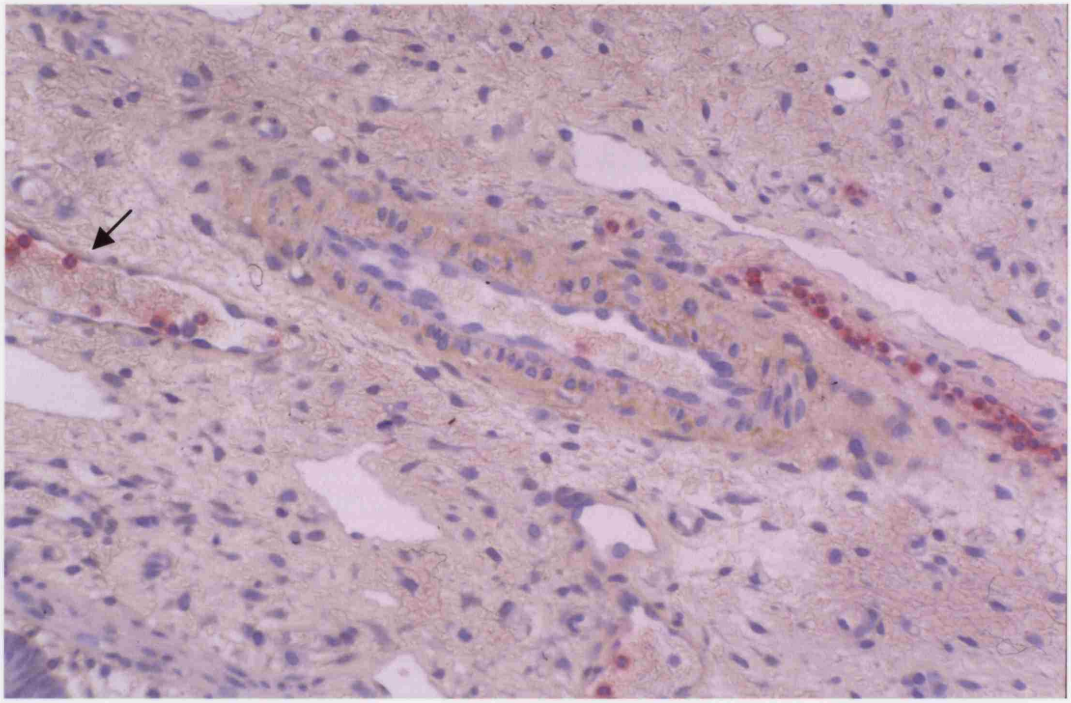


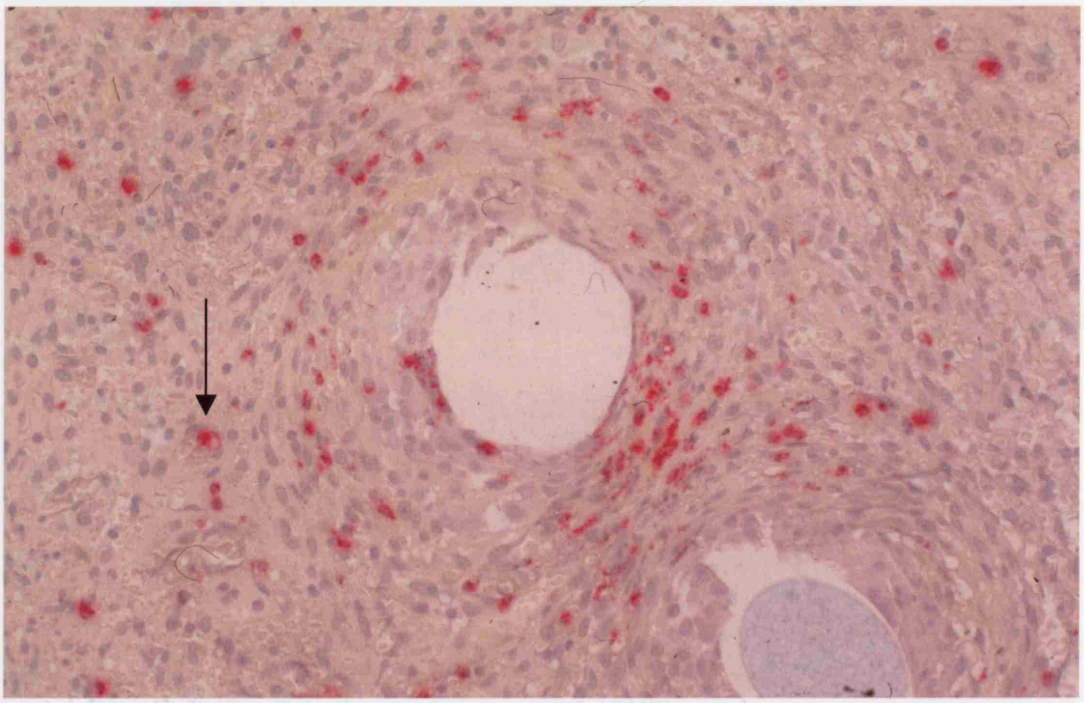
**Figure 12** Localised breakdown in the continuity of endothelial cell glycosaminoglycans (arrow) in relation to neutrophil adhesion (original magnification x 10).



### 4.3.3 Peri-cellular matrix glycosaminoglycans not directly degraded by inflammatory cells

Within the different groups of disease severity, there was a consistent pattern of GAG distribution in the sections examined in relation to inflammatory cells. Conversely, there was no demonstrable spatial relationship between extra-vascular GAG attenuation and the intensity of the cellular infiltrate. This was true in the mucosa and serosa but was most easily verified in the peri-cellular matrix of the sub-mucosa (Figure 13).

**Figure 13** Neutrophils in the peri-vascular matrix of the submucosa in moderate necrotizing enterocolitis. There is no surrounding halo of glycosaminoglycan degradation (original magnification x 40).



## 4.4 Discussion

Degradation of GAGs is well described in a variety of tissues in both experimental and human studies (McGowan and Thompson, 1989) (Klebanoff et al., 1993). The contribution of neutrophils and macrophages to this process has been particularly well studied. They operate by the generation of reactive oxygen species (ROS) and the secretion of glycanases and have been shown to degrade the major components of GAGs (Matzner et al., 1992) (Moseley et al., 1997) (Matzner et al., 1985). A few studies report the modulation of GAGs in intestinal disease but there are no reports correlating GAG attenuation with the inflammatory infiltrate in NEC.

Degradation of GAGs during inflammation may have a profound effect on the inflammatory cascade and it's consequences. For example, GAGs are thought to have a dual role in neutrophil migration. In addition to promoting diapedesis (Matzner et al., 1985), they may inhibit neutrophil activity by reducing the generation and release of ROS, lysozyme, beta-glucuronidase and platelet activating factor (Bazzoni et al., 1993) (Capecchi et al., 1993, Labrouche et al., 1992). In addition, they may protect local tissue from damage by enzymes released as the cells migrate

(Webb et al., 1993), the overall effect being a substantial anti-inflammatory role. The loss of GAGs in NEC may contribute to a cascade in which a reduction of the anti-inflammatory role of GAGs promotes the inflammatory process and the consequences of loss including excessive protein leakage (Klebanoff et al., 1993) with progression to CLS and related complications.

This series of experiments has confirmed GAG degradation in NEC proportionate to histological disease severity. The breakdown of endothelial GAGs at the point of neutrophil and macrophage adherence has also been demonstrated. Unexpectedly, there appeared to be no correlation between peri-cellular matrix GAG degradation and the intensity of neutrophil and macrophage infiltration, suggesting that the patho-physiological mechanisms underlying the majority of GAG loss in NEC differs from that previously shown in ulcerative colitis and Crohn's disease (Murch et al., 1993). This emphasises again that although there are several overlapping features amongst the enterocolitides, each one is a distinct entity, with pathophysiological as well as clinical peculiarities.

In order to study the role of the inflammatory infiltrate further, the APAAP technique was utilised with an extended panel of monoclonal antibodies. Antibodies against antigens carried by neutrophils, monocytes, macrophages, lymphocytes and a group of adhesion molecules were employed. The results of these experiments are presented in Chapter 5.

# Chapter 5

# The inflammatory response in neonatal necrotizing enterocolitis; an immunohistochemical analysis

## 5.1 Introduction

General histopathological findings in NEC are well recognised. Typical features include haemorrhage, lymphatic dilatation, a mixed ICI, mucosal ulceration and necrosis (Ballance et al., 1990). In a minority of cases a paucity of inflammatory cells is described (Joshi, 1978). Assumptions of the importance of the ICI in NEC are not backed by scientific data as attempts to characterise the cells in detail were previously limited by an inability to reliably distinguish between them.

Advances in immunohistochemical techniques have made detailed examination of the ICI possible (Crocker et al., 1984) (Kramps et al., 1984) (Pulford et al., 1988) (Fell et al., 1996). Despite the widespread availability of the techniques, there are still no studies that have utilised specific cellular markers to characterise, in detail, the cellular infiltrate in NEC. This chapter reports a series of experiments designed to characterise the ICI in NEC.

## 5.2 Methods

### 5.2.1 Patients

Tissue from the group of patients described in Chapter 4 was utilised; 20 infants who underwent intestinal resection and a primary anastomosis in which the proximal resection involved small intestine. The median gestational age was 28 weeks and birth weight 1220g. At the time of surgery, the median age of the infants was 15 days and 14 (70%) out of 20 had sustained intestinal perforation(s) (Table 6). Details of surgery and outcomes have been reported (Ade-Ajayi *et al.*, 1996a). Control sections (n=5) were taken from intestinal resection specimens of infants with ileal atresia which did not have any histological abnormalities demonstrable on H&E sections.

### 5.2.2 Tissue preparation

Tissue sections were prepared as previously described in Chapters 2 and 4. In brief, intestinal specimens from the proximal resection margin were placed in 10% formalin and embedded in paraffin. Three micron sections were cut from the paraffin blocks and mounted on Vectabond coated slides

(Vector laboratories SP1800). The sections were dewaxed and, in addition, sections for CD68, CD3 and CD20 immunohistochemistry were subjected to high temperature antigen unmasking (Cattoretti et al., 1993).

**Table 6 Findings in infants who underwent resection for NEC**

| No. | Sex | Gestation | B. Wt. (g) | Age (days) | Laparotomy findings | Overall histological severity score |
|---|---|---|---|---|---|---|
| 1 | F | 26 | 1174 | 35 | Gangrene, | 4 |
| 2 | F | 25 | 750 | 42 | NEC, stricture | 9 |
| 3 | M | 28 | 1190 | 22 | Gangrene | 12 |
| 4 | M | 33 | 1660 | 13 | Gangrene | 8 |
| 5 | M | 38 | 2608 | 30 | Gangrene, | 9 |
| 6 | M | 25 | 820 | 17 | Gangrene, | 4 |
| 7 | M | 25 | 940 | 11 | Perforation | 12 |
| 8 | F | 31 | 1380 | 46 | Perforation | 11 |
| 9 | F | 28 | 516 | 9 | Perforation | 12 |
| 10 | M | 38 | 2980 | 3 | Gangrene, | 6 |
| 11 | M | 26 | 1400 | 6 | Gangrene, | 8 |
| 12 | M | 27 | 1079 | 96 | NEC | 9 |
| 13 | F | 28 | 705 | 20 | Gangrene | 11 |
| 14 | M | 39 | 3400 | 5 | Gangrene | 9 |
| 15 | M | 37 | 2300 | 9 | Gangrene, | 12 |
| 16 | M | 30 | 1526 | 12 | Perforation | 6 |
| 17 | F | 28 | 770 | 24 | Perforation | 10 |
| 18 | M | 37 | 2100 | 2 | Gangrene, | 6 |
| 19 | M | 29 | 1250 | 8 | Perforation | 5 |
| 20 | F | 26 | 870 | 46 | Perforation | 8 |

Abbreviations: NEC, necrotizing enterocolitis without necrosis or perforation; B. Wt., birth weight; gangrene, gangrenous bowel.

### 5.2.3 Haematoxylin and eosin

Haematoxylin and Eosin stains were carried out as previously described.

### 5.2.4 APAAP immunohistochemistry

For leucocyte antigen localisation, the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was employed with an extended panel of monoclonal antibodies (Table 7). Primary mouse monoclonal antibody was applied (dilution 1:50) and incubated for 30 minutes. Rabbit anti-mouse immunoglobulin (1:25 dilution - DAKO Code no. Z 0259) and APAAP mouse monoclonal complex at a dilution of 1:50 (DAKO Code no. D0651) were each incubated for 30 minutes with intervening 5 minute washes using Tris buffered saline (TBS) in between. A fast red alkaline phosphatase substrate was then applied for 20 minutes and the reaction terminated in water. Meyer's Haematoxylin was used as a counterstain and the slides mounted in Aquamount (BDH, Essex, UK).

The expression of the adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) were also

determined following high temperature citration. Goat polyclonal antibody was applied to the slides at a dilution of 1:20 followed by incubation in a moist chamber for 90 minutes. A 5 minute wash in TBS was then followed by incubation with monoclonal antibody IgG. Fast red and Meyer's haematoxylin were applied as previously described.

**Table 7 Extended panel of monoclonal and polyclonal antibodies with specifications**

| Antibody | Antigen | Spec. | Main cellular expression |
|---|---|---|---|
| * Anti-human neutrophil elastase | Neutrophil elastase (clone NP57) | Dako M0752 | N |
| * Anti-human macrophage | clone KP1 (CD-68) | Dako MO814 | Mac |
| * Anti-human myeloid/histiocyte | L1/ Calprotein (MAC 387) | Dako M0747 | N, Mac, Mon |
| * Anti-human T cell | Clone UCHT1 (CD-3) | Dako E7007 | T cells |
| * Anti-human B cell | Clone B-Ly1 (CD20) | Dako M0774 | B cells |
| * Anti-human HLA-DR | Clone DK22 (HLA-DR) | Dako M0775 | Mac, B cells, E |
| * Anti-human Ki-67 | Ki-67 | Dako A047 | Proliferating cells |
| **Anti-human ICAM-1 | ICAM-1 (CD 54) | R&D BBA17 | Activated E |
| **Anti-human E – Selectin | E – Selectin (CD62E) | R&D BBA18 | Activated E |
| **Anti-human VCAM-1 | VCAM-1 (CD 106) | R&D BBA19 | Activated E |

Abbreviations: Spec., specifications; *, monoclonal; **, polyclonal; N, neutrophil(s); Mac, macrophages; Mon, monocytes; E, endothelial cells.

### 5.2.5 Extravidin Biotin Peroxidase technique

In order to confirm observations of lymphocyte patterns, the peroxidase method of antigen localisation was utilised to detect CD3 (T-lymphocytes) and CD20 (B-lymphocytes) in a sub-group of infants (n = 5). Briefly; endogenous peroxidase was blocked with 10% hydrogen peroxidase in PBS. Following high temperature antigen unmasking, the slides were incubated with primary antibody for 1 hour and then secondary antibody for a further hour. Incubation in Extravidin for another hour was then followed by development of peroxidase activity over 10 minutes and counterstaining with Meyer's haematoxylin.

### 5.2.6 Histological examination

An Olympus BH-2 light microscope was used. Overall assessment of each section at low and high power magnification was carried out for descriptive purposes. In addition, high power fields of epithelium, submucosa and serosa were analysed semi-quantitatively (Ade-Ajayi et al., 1996b). Briefly, in sections stained with H&E, each layer was scored for disease severity using haemorrhage, ulceration, architectural destruction and necrosis as parameters and sections were classed as being well

preserved, moderately diseased and severely diseased. The term histological disease severity is used in the text to refer to this classification.

### 5.2.7 Neutrophil elastase positive cell counts

The degree of infiltration with neutrophil elastase positive cells, was quantified in each complete transverse section (n =10 ) by counting the number of positive cells in each of 30 high power fields (10 segments of serosa, submucosa and mucosa). The mean of two counts was taken as representative of each field. Each segment was graded for histological disease severity using the criteria described above. Data sets were grouped into those from well-preserved, moderately and severely affected fields.

## 5.3 Results

### 5.3.1 Wide range of disease severity

The diagnosis of NEC was confirmed histologically in the 20 neonates with a wide range of disease severity demonstrable on H&E sections. On overall assessment at the resection margin, histological disease severity score placed the sections of 2 infants in a mild group with very good architectural preservation, slight lymphatic dilatation and mild haemorrhage (score $\leq 4$). Seven infants had a moderate score (5-8) and 11 had severe changes with histological evidence of widespread ulceration, necrosis and architectural disruption (Table 6).

### 5.3.2 Granulocyte patterns in mild and severe disease

In infants with mild disease on overall assessment of sections, the majority of neutrophil elastase positive cells were confined to the serosa (Figure 14 a). With an increase in histopathological severity score to moderate, neutrophil elastase positive cells were evident in submucosal blood vessels (Figure 14 b). The neutrophil distribution was similar to that of newly

recruited macrophages and in severe disease, numerous granulocytes were evident in the mucosa. Anti-CD68 was used to determine macrophage distribution. In the main, CD 68 positive cells followed the pattern of progression observed with neutrophils. In addition, MAC 387, which provided a particularly robust stain, confirmed the distribution of neutrophils, monocytes and newly recruited macrophages. These were mainly in the periphery until the onset of mucosal ulceration when there was heavy infiltration of the mucosa. Granulocyte infiltration of the mucosa is demonstrated in Figure 14 c (severe NEC), with cytoplasmic staining within MAC-387 positive cells.

The number of NE positive cells ranged from 0 to 450 per high power field. The segmental distribution of NE positive cells in mild and severe disease is illustrated graphically in Figures 15 and 16.

**Figure 14** (a) Photomicrograph of a histological section from the proximal resection margin of an infant with necrotizing enterocolitis following neutrophil elastase staining (original magnification x 2.5). Intestinal architecture is preserved and the majority of neutrophils are confined to the serosa. (b) In moderate disease, neutrophils are evident in submucosal blood vessels (arrow) and to a limited extent in the mucosa (original magnification x 2.5). (c) In severe NEC, granulocyte infiltration of the mucosa is shown by cytoplasmic staining within MAC-387 positive cells (macrophages, neutrophils and monocytes) in the ulcer.

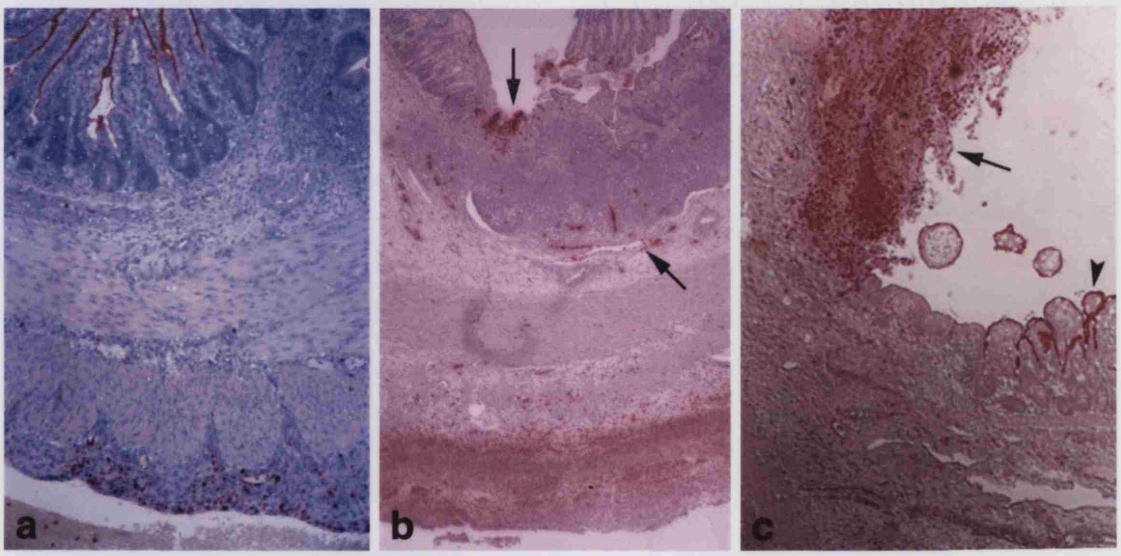
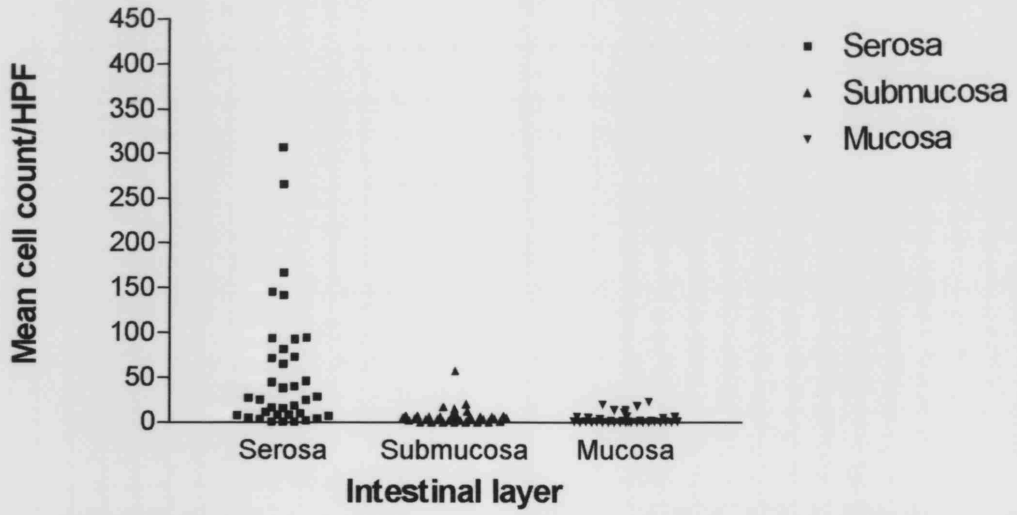


**Figure 15 Graph of segmental neutrophil cell counts in mild NEC**

**Figure 15** Neutrophil elastase positive cell counts in well-preserved segments of small bowel from the resection margins of infants with NEC. The neutrophils are predominantly within the serosa.



### 5.3.3 Prominent lymphoid aggregates with evidence of activation

Lymphoid aggregates were present and prominent in 18 of the 20 patients. Of 86 discrete lymphoid aggregates counted, 52 (60%) were subjacent to an area of mucosal ulceration (Figure 17). These aggregates consisted of CD3 and CD20 positive cells. The initial observations were made following APAAP immunohistochemistry and were confirmed in a sub-group of infants employing the Peroxidase technique (n=5). In mild disease, the lymphoid aggregates were distinct and consisted of CD20 cells in the centre with CD3 cells in the periphery of each aggregate. In more severe disease, the spatial distinctions between the two cell types were blurred and the number of CD3 cells relative to CD20 appeared to increase (Figure 18).

**Figure 17** Prominent lymphoid follicle subjacent to an area of mucosal ulceration in severe necrotizing enterocolitis (H&E stain, original magnification x 10).

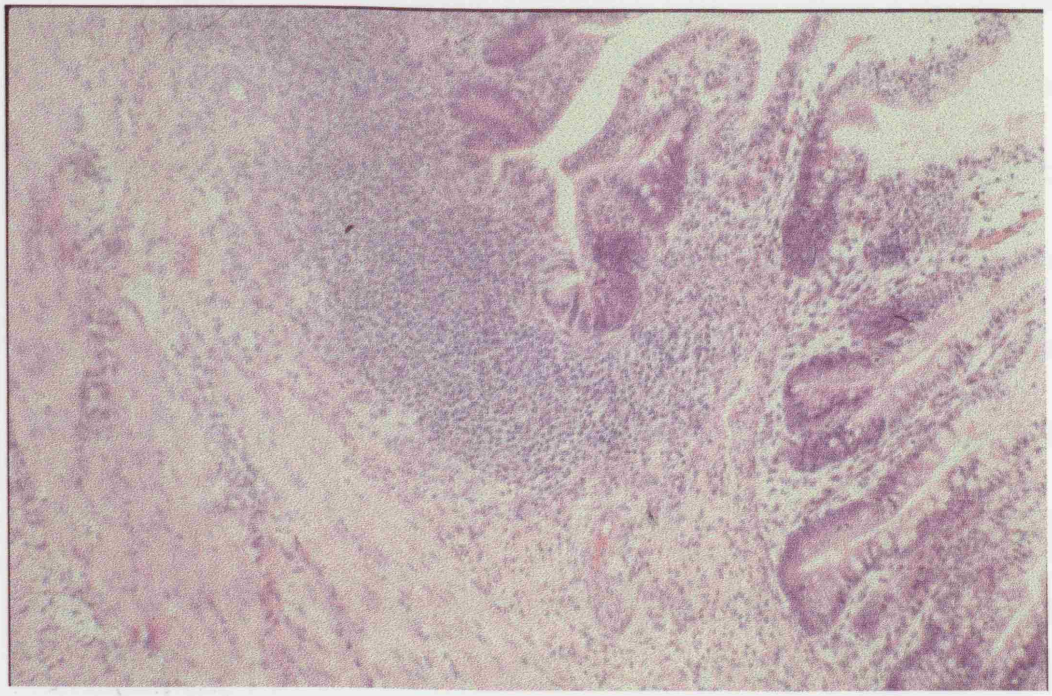
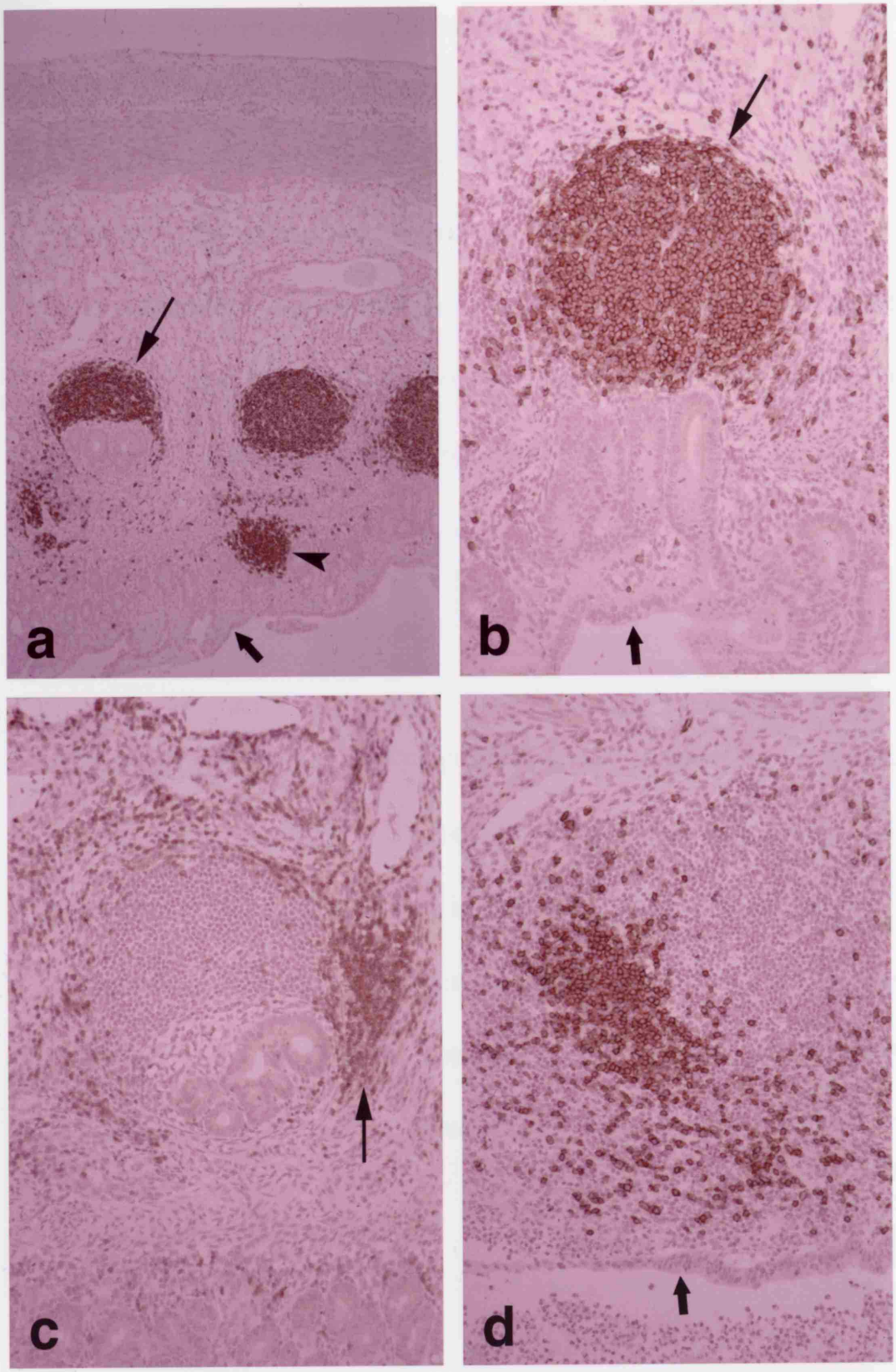


**Figure 18** (a) CD20 positive B-lymphocytes (brown stain) within lymphoid aggregates in the mucosa and submucosa of intestine affected by mild necrotizing enterocolitis (NEC) (original magnification x 4). (b) CD20 positive B-lymphocytes in intestine with moderate necrotizing enterocolitis (original magnification x 10). (c) CD3 positive T-lymphocytes on the periphery of a lymphoid aggregate in the submucosa (arrow) and within the mucosa of bowel affected by moderate necrotizing enterocolitis (original magnification x 10). (d) Section of intestine affected by severe NEC with mucosal thinning (arrow). There is an increasing proportion of T cells relative to the remainder of cells within the lymphoid aggregate as demonstrated by CD3 stain (original magnification x 10).


a
b
c
d

### 5.3.4 Endothelial cell activation

HLA-DR was expressed on endothelial cells. This was maximal in vessels located in the submucosa of sections with severe NEC. ICAM-1 expression was also evident in the endothelium of vessels in both the submucosa and serosa (Figure 19). Furthermore, there was upregulation of VCAM-1 and E-selectin at the same sites in severe disease.

### 5.3.5 Cellular activation

Using Ki67 as a marker of proliferation, the overall degree of cellular proliferation was higher in infants with NEC than within control tissue. This was particularly true within lymphoid aggregates where the proportion of Ki67 positive lymphoid cells approached 80% in severely affected bowel compared to control bowel in which only about 20% of lymphocytes were Ki67 positive (Figure 20).

**Figure 19** Demonstrating up-regulation of (a) HLA-DR and (b) ICAM-1. Arrows highlight endothelial cell activation which is most prominent in the submucosa (original magnification x 40).

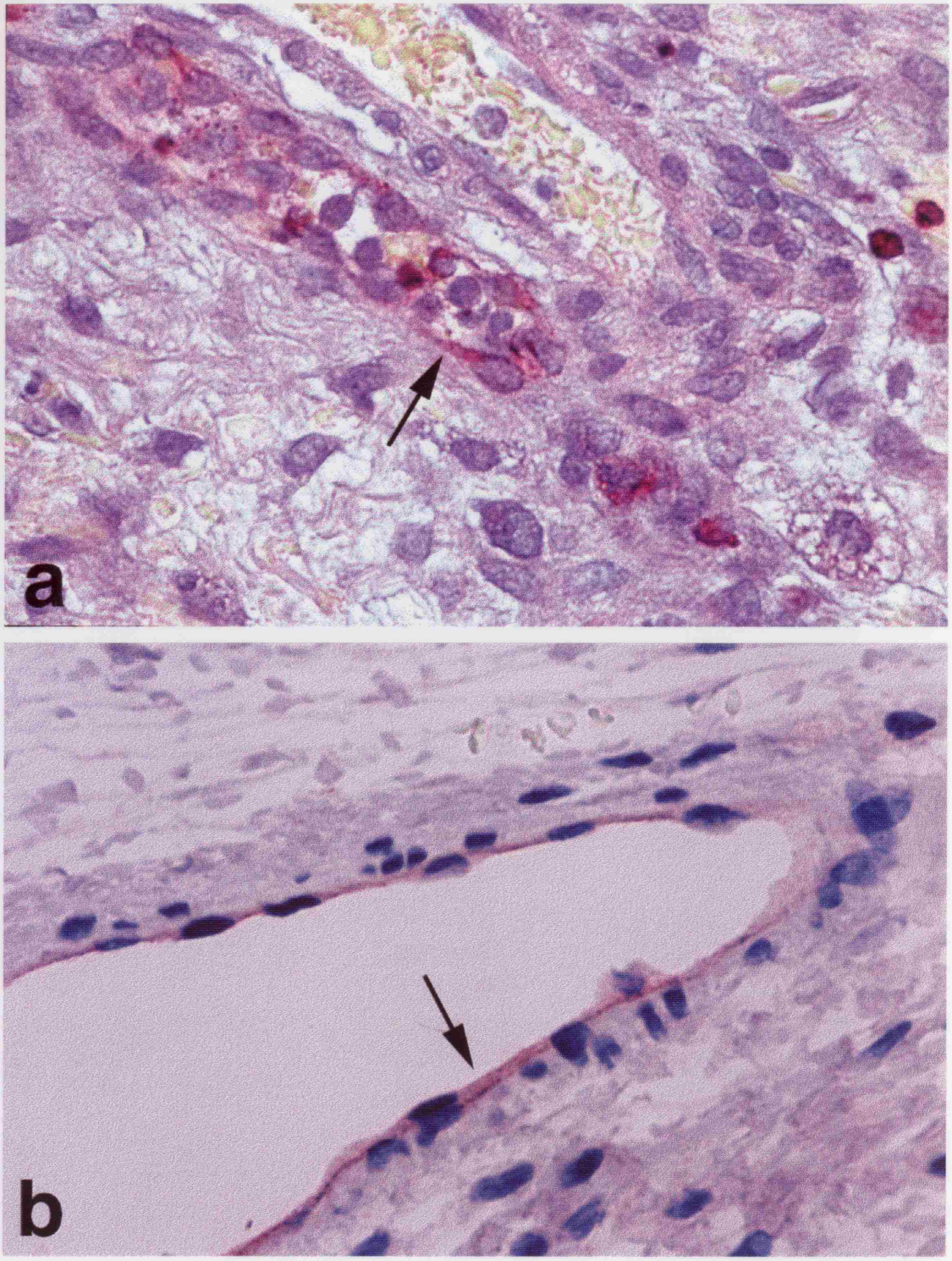
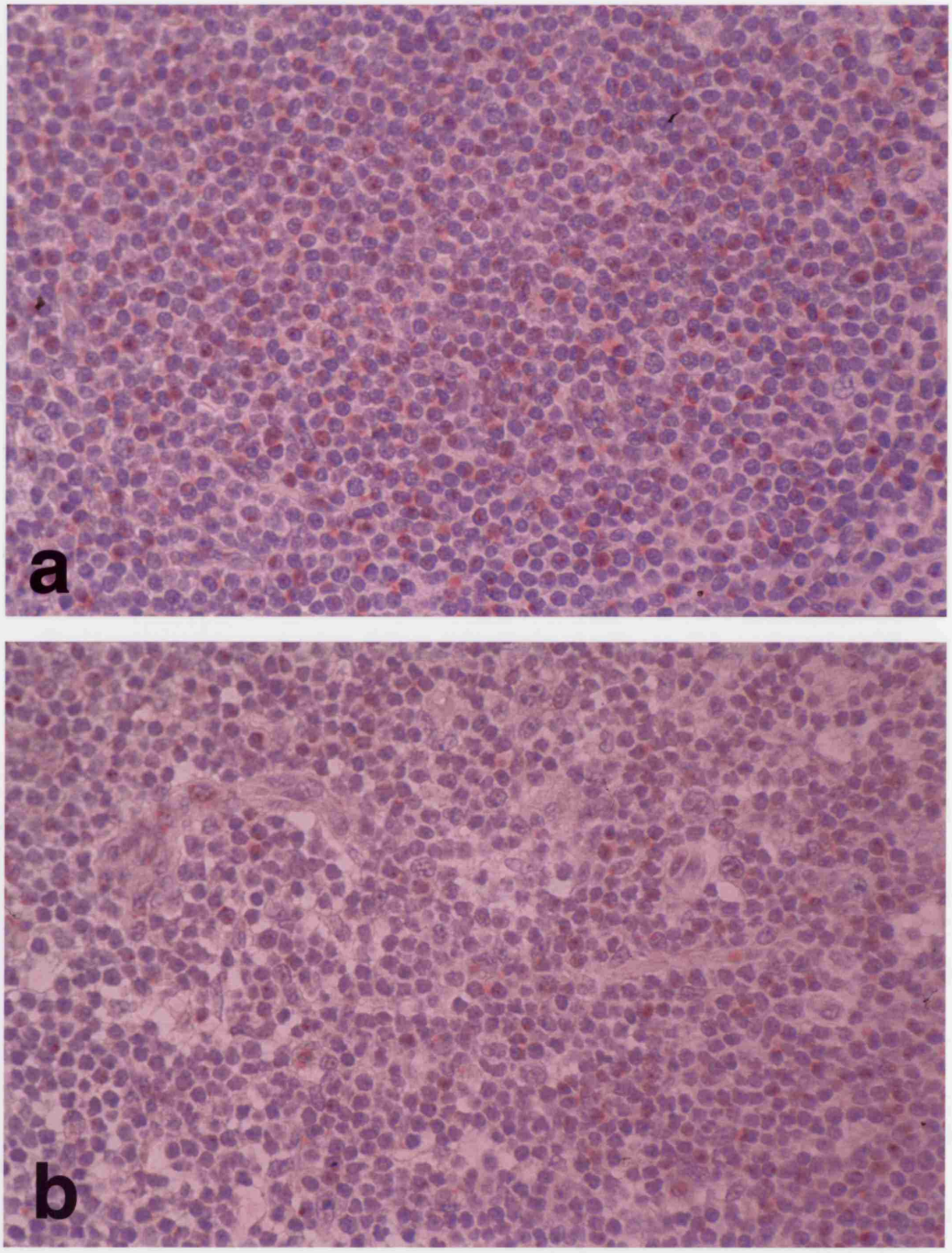


**Figure 20** High power view of prominent lymphoid aggregate subjacent to an area of mucosal ulceration (original magnification x 40). (a) The majority of cells were found to be Ki67 positive, compared to control tissue, (b), in which a minority of cells expressed the antigen.



## 5.4 Discussion

There is increasing evidence of the role of inflammatory cells and mediators in the pathophysiolgy of a range of inflammatory disorders of the bowel (Dupont and Heyman, 2000), (Shiner, 1982), (Takeda et al., 1999), (Song et al., 1999), (Lowes et al., 1992). Although NEC is a distinct disease entity, there are some shared characteristics with other enterocolitides and it seems likely that the pathophysiology is also modulated in part by inflammatory cells and their products (Figure 21).

Insight into typical histopathological features of NEC was provided by Ballance who examined intestinal tissue from 84 patients with NEC (Ballance et al., 1990). Ischaemic necrosis and inflammation were the most prevalent histologic findings. There was a slight preponderance of acute inflammatory changes and serositis was described in 57 infants and trans-mural inflammation in 42. Correlation with degree of architectural disruption was not provided. It was concluded that the histologic changes of NEC suggest a multi-factorial cause, with ischaemia and inflammation playing important roles. Direct comparisons with the experiments reported here are not valid primarily because no attempt was made to correlate the inflammatory infiltrate with intestinal architecture in that study. However,

the descriptive report of acute and chronic inflammation, haemorrhage, ulceration, evidence of reparative changes and oedema is consistent with our own findings. In another clinico-pathological study, Tait reported infants who underwent bowel resection for NEC and described a mixed inflammatory infiltrate consisting of lymphocytes, neutrophils, plasma cells, histiocytes and occasional mast cells (Tait and Kealy, 1979). The infiltrate was maximal in the submucosa but present in lesser amounts in the muscularis propria and serosa. There was no grading of histological disease severity. A previous report has drawn attention to the paucity of the inflammatory cells in early NEC lesions in comparison to more advanced lesions with denuded mucosa (Santulli et al., 1975). This is a finding that we have confirmed in this report and gone on to characterise in detail.

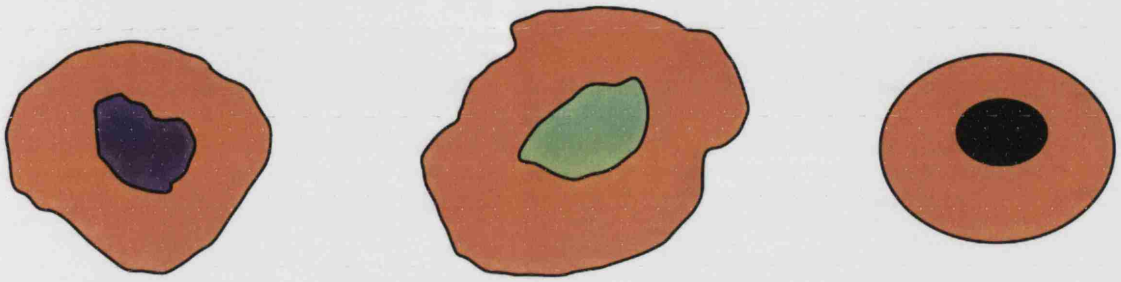
In many reports, examination of the inflammatory infiltrate in NEC has focused on neutrophils. Often, evidence of activity has been based on neutrophil products such as myeloperoxidase rather than morphological data. For example, Musemach et al induced neutropenia in rats using Vinblastine and documented a reduction in myeloperoxidase activity and intestinal necrosis in treated animals when compared with controls (Musemeche et al., 1991). They concluded that neutrophils and their

products play a major role in the pathogenesis of NEC. Lymphocytes were not examined. In another study in which myeloperoxidase levels were used as an index of neutrophil activity, Langer found no difference between animals who had undergone IR and sham controls. They concluded that neutrophils do not mediate the *early* increase in mucosal permeability after IR (Langer et al., 1995). Despite the aforementioned, it is widely assumed that neutrophils are central to the events that occur shortly after IR in NEC. There is therefore a need for comprehensive information regarding the distribution and role of components of the ICI in NEC.

The material used for a study of this nature is important. Central portions of bowel resected from infants with advanced NEC is often necrotic. Use of such material for morphological analyses may be unsatisfactory in view of the severity of architectural disruption that has occurred. In these experiments, we have examined the proximal resection margins involving small bowel. This has allowed standardisation and provided tissue demonstrating a range of degrees of histological severity from well preserved to severely affected. Changes visualised have been referred to as mild, moderate and severe NEC, terms that describe morphological changes and as such are primarily *histopathological.* Pathophysiological processes, particularly leucocyte responses, are inferred from the

observations. The nature of an immunohistochemical study is that it is primarily descriptive. However, clarity of the neutrophil elastase stain, and the consistent pattern of infiltration in relation to the segmental architecture of the sections, enabled quantification to be carried out by cell counting.

**Figure 21** Examples of cellular products involved in inflammation.



| Neutrophil | Macrophage | Lymphocyte |
|---|---|---|
| myeloperoxidase | IFN-γ | IFN-γ |
| collagenase | IFN-β | IFN-α |
| elastase | TNF-α | TNF-α |
| cathepsins | TNF-β | TNF-β |
| leukotriene B4 | G-CSF | IL-2 |
| | Prostaglandin E2 | IL-6 |
| | Prostaglandin F2 | IL-8 |
| | IL-1 | |
| | IL-6 | |
| | IL-8 | |

IFN, interferon; TNF, tumour necrosis factor, G-CSF; I-L, interleukin.

The first of our main findings was that in early *histopathological* NEC, neutrophil elastase positive cells were restricted to the serosa. Only promyelocytes and more differentiated myeloid cells stain positive for elastase (Kramps et al., 1984), therefore these cells are regarded as neutrophils and referred to in this report as such.

As 70% of the patients studied had a bowel perforation at the time of laparotomy, serosal inflammation secondary to peritonitis was considered as an explanation for this phenomenon. In our study, with increased histological severity, the mucosa underwent infiltration with neutrophils. As luminal infiltration occurred, there was a trend towards reduction in the number of cells in the abluminal surface, suggesting that the serosal neutrophil response in early disease was part of an organised process of recruitment, not merely the result of peritonitis.

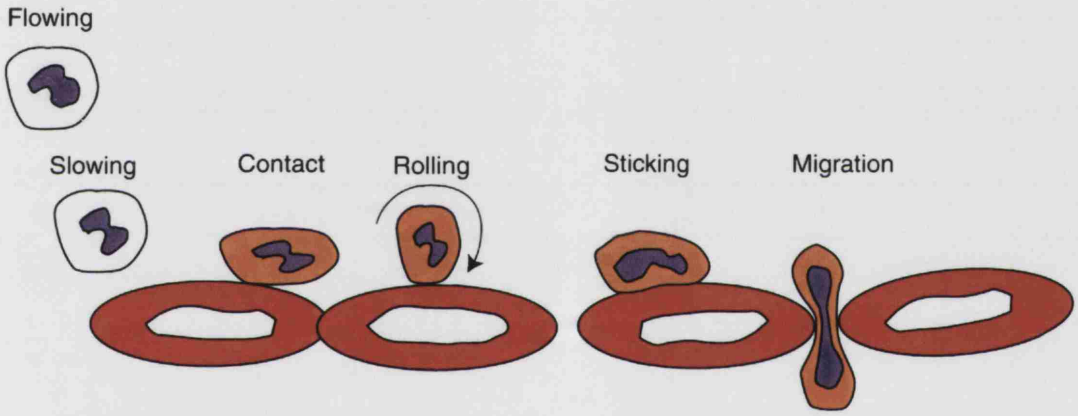
In one of the few clinical studies that refer to neutrophil recruitment in NEC, Balcom reported substantial mobilisation of mature neutrophils into the peritoneum associated with peripheral neutropenia in 7 infants with the disease (Balcom et al., 1985). This mobilisation was associated with

neutropenia in human infants and depletion of bone marrow stores in experimental animals. They did not, however, provide a description of trans-mural neutrophil distribution. In addition, the wide range of disease severities encountered in NEC, was not addressed.

The process of neutrophil recruitment to sites of inflammation is a complex one, involving a number of inter-related mechanisms. It occurs in response to hypoxia and re-oxygenation, and involves other inflammatory cells and secondary mediators of inflammation (Colgan et al., 1996) (Kanwar and Kubes, 1994). At the local site of inflammation, neutrophil endothelial passage is mediated by adhesion molecules (Steadman et al., 1997) via a multi-step mechanism which includes rolling, activation, sticking, and endothelial trans-migration (see Figure 22).

Large macrophages immunoreactive for CD-68 and MAC-387 were found in a similar distribution to neutrophils. Calprotectin expression in macrophages is a marker of recent recruitment. It is substantially increased within the mucosal macrophage population in active inflammatory bowel disease (Rugtveit et al., 1994) and it's observation here represents another example of the overlap between different enterocolitides.

**Figure 22** Diagram depicting events leading to trans-endothelial neutrophil migration.



The intestine is the largest and most complex lymphoid organ in the body and the interactions which determine it's responses to a range of external stimuli are still in the process of characterisation. In particular, factors that control mucosal T cell activation and suppression are poorly understood (Abreu-Martin and Targan, 1996). The degree of lymphoid activity in this study was an unexpected finding. The presence of large lymphoid aggregates erupting into areas of mucosal ulceration was striking. A high degree of Ki67 activation in lymphoid follicles of infants with NEC, compared with controls, was consistently demonstrated. Taken in isolation, the significance of this degree of activation is unclear as human foetal intestine has previously been shown to have significant Ki67 activity (Howie et al., 1998). However, further evidence of the importance of these observations is provided by the marked expression of HLA-DR in vascular endothelium. Cytokines responsible for HLA-DR induction have been characterised and include interferon γ (IFNγ) and TNF (Lowes et al., 1992, Ishii et al., 1994),(Sturgess et al., 1992).

It has long been known that lymphocytes may be major contributors to the pathologic process of enteropathy. Exploring the effects of

immunologically mediated intestinal damage in a mouse model of allograft rejection, McDonald demonstrated lymphocytic infiltration of the lamina propria, crypt hyperplasia and mucosal ulceration. Interestingly, similar to the findings in this study, there was no lamina propria infiltration of neutrophils prior to mucosal ulceration (MacDonald and Ferguson, 1976). A lymphokine linking the activated T cells to mucosal destruction was suggested. This "lymphokine" has been better characterised since that study.

We now know that T lymphocytes are rich sources of mediators such as the interleukins 1-6, IFN, TNF, granulocyte macrophage colony stimulating factor (GM-CSF) and transforming growth factor beta (TGF beta) (Feldmann et al., 1989). Liesenfeld recently demonstrated the importance of T cell mediated activation of tumour necrosis factor (TNF) and IFNγ in the development of small bowel necrosis in susceptible mice following intestinal infection (Liesenfeld et al., 1999). Furthermore, in foetal intestinal organ culture, lymphocyte activation induces destructive enteropathy due to macrophage-mediated matrix disruption. The central role of TNF α in this process is demonstrated by prevention of tissue damage with TNF blockade (Pender et al., 1996, Pender et al., 1998).

Cytokine induction is pivotal in the pathophysiology of shock (Giroir, 1993). Understanding the events associated with the release of these secondary inflammatory mediators is central to efforts to develop novel therapies for the prevention and treatment of MSOF in NEC. TNF α is one of a number of important mediators in the immune response to infection. It plays a key role in the events leading up to and following the systemic inflammatory response syndrome (SIRS). It has been suggested that the sequence of events include an initial stage of local cytokine production, a second stage involving systemic release of small amounts of cytokine and a third in which large amounts of systemic cytokines are released (Bone, 1996). The consequences of this exaggerated cascade include intra-vascular coagulopathy, defects in cell adhesion and vascular as well as end organ damage (Beutler and Grau, 1993).

Direct scientific evidence for the involvement of secondary mediators in infants with NEC is not as strong as in other disease models. Morecroft did not demonstrate an association between circulating levels of TNF and NEC severity (Morecroft et al., 1994b). Tissue distribution of TNF was not examined in that particular report. Harris also examined TNF plasma levels in critically ill infants with sepsis and NEC (Harris et al., 1994). He was also unable to consistently demonstrate elevation of TNF when these

infants were compared to controls. In that study, IL-6 was a more reliable indicator of disease severity. It is possible that TNF is a less reliable marker because of it's short half life.

Despite the above, there is some evidence linking inflammatory mediators to NEC. The administration of TNF and PAF to animals produces NEC like lesions experimentally (Kliegman, 1990). In addition, Ford demonstrated increased expression of nitric oxide and IFNγ in the intestine of infants with NEC (Ford et al., 1996). These findings are consistent with the upregulation of HLA-DR demonstrated in this study.

Further evidence for the role of secondary inflammatory mediators is provided by other experimental studies. Recently, it was observed that immature human enterocytes in a cell line (H4), reacted with excessive pro- inflammatory cytokine production following inflammatory stimulation with lipopolysaccharide LPS and interleukin 1 beta when compared with mature human small intestine (Caco-2 cell line). Interestingly, the sites of excessive activity were found to be in villous and crypt epithelium and immuno-responsive lymphoid cells (Nanthakumar et al., 2000).

Upregulation of VCAM-1 and ICAM-1 was a consistent finding in this study. The expression of these antigens is unlikely to be developmental as control sections did not show similar expression. Adhesion molecules are involved in a wide range of pathophysiological processes. They are particularly important with regard to the mechanisms regulating adhesive interactions between blood cells and the vessel wall. These activities depend on the activation of receptors present on the vessel wall or expression of new receptors on the cell surface (Frenette and Wagner, 1996b, Frenette and Wagner, 1996a). VCAM-1 plays an especially important role in the process of lymphocyte migration.

The findings of prominent lymphocytic aggregates related to areas of ulceration, evidence of lymphocytic proliferation and upregulation of HLA-DR and adhesion molecules suggest an immunological component that is central to the inflammatory cascade. The data is consistent with an exaggerated response to an antigenic load and it is conceivable that cows milk protein, microbes and microbial products constitute such a load.

The question of potential clinical application arises from a study of this nature. Host immunological responses are clearly important in NEC and

increased IgA levels have been detected in infants with the disease when compared with controls (Bell et al., 1985). The encouragement of breast feeding whenever possible is appropriate first line "immunotherapy" as this provides immunoglobulins, macrophages and lymphocytes and avoids putative allergens in cow's milk. In order to advocate pharmacological manipulation of immunologic mechanisms, more information is required. For example is there a particular exaggeration of the lymphocyte response in those infants with NEC who run a fulminant disease course compared to those with a more benign course? What are the T cell subsets most likely to be activated in severe disease?

## 5.5 Conclusions

This study relates inflammatory cell distribution in NEC with morphological evidence of disease severity based on well-defined histopathological indices. In mild histopathological NEC, the active granulocytic component of the inflammatory response was largely confined to the serosa. Infiltration of the mucosa with neutrophils and newly recruited macrophages was demonstrated with mucosal ulceration. Lymphoid aggregates were prominent and displayed a high degree of activation. Endothelial cells in submucosal vessels showed evidence of activation with expression of HLA-DR, ICAM-1, VCAM-1 and E-

Selectin. Control vessels did not express these antigens. The nature of endothelial activation with HLA-DR, ICAM-1 and VCAM-1 expression is consistent with the presence of lymphocyte derived cytokines.

While much attention has focused on the role of neutrophils in NEC our data indicates that lymphocytes also play an important role in the inflammation associated with this condition. The finding that activated lymphoid follicles were immediately subjacent to ulcerated epithelium suggests an important analogy to better recognised cell-mediated immuno-pathologies such as Crohn's disease, in which the initial ulceration occurs over lymphoid follicles (Murch, 1999). The presence of villous atrophy adjacent to ulcerated areas is further evidence of adaptive immune responses, as this tissue response occurs as a specific consequence of T cell activation (Book et al., 1976).

It is evident from *in vitro* organ culture of human foetal small intestine that therapeutic inhibition of T cell activation with corticosteroids or cyclosporine/tacrolimus can prevent ulceration and tissue destruction through matrix degradation (Book et al., 1976). This raises the question of whether there may be a role for specific therapeutic inhibition of T cell

activation in infants with early or suspected NEC, in which similar matrix degradation occurs. Pentoxifylline is a methylxanthine derivative with anti TNF properties. It has been used to treat premature new-born sepsis with demonstrable reduction in TNF levels and a reduced mortality in infants on treatment compared with placebo (Lauterbach and Zembala, 1996, Lauterbach et al., 1999). Interventions such as this may, in the future, have a role in the treatment of NEC.

We suggest that a major pathophysiological component in NEC is a lymphocyte driven, cytokine mediated, intestinal immunopathy in which lymphocyte activation occurs in response to an antigenic load. Further studies may guide pharmacological manipulation directed at these immunological mechanisms. Whether this will result in a reduction of the high morbidity and mortality currently associated with NEC remains to be seen.

# Chapter 6

# Urinary glycosaminoglycan levels in acute intestinal ischaemia with reperfusion

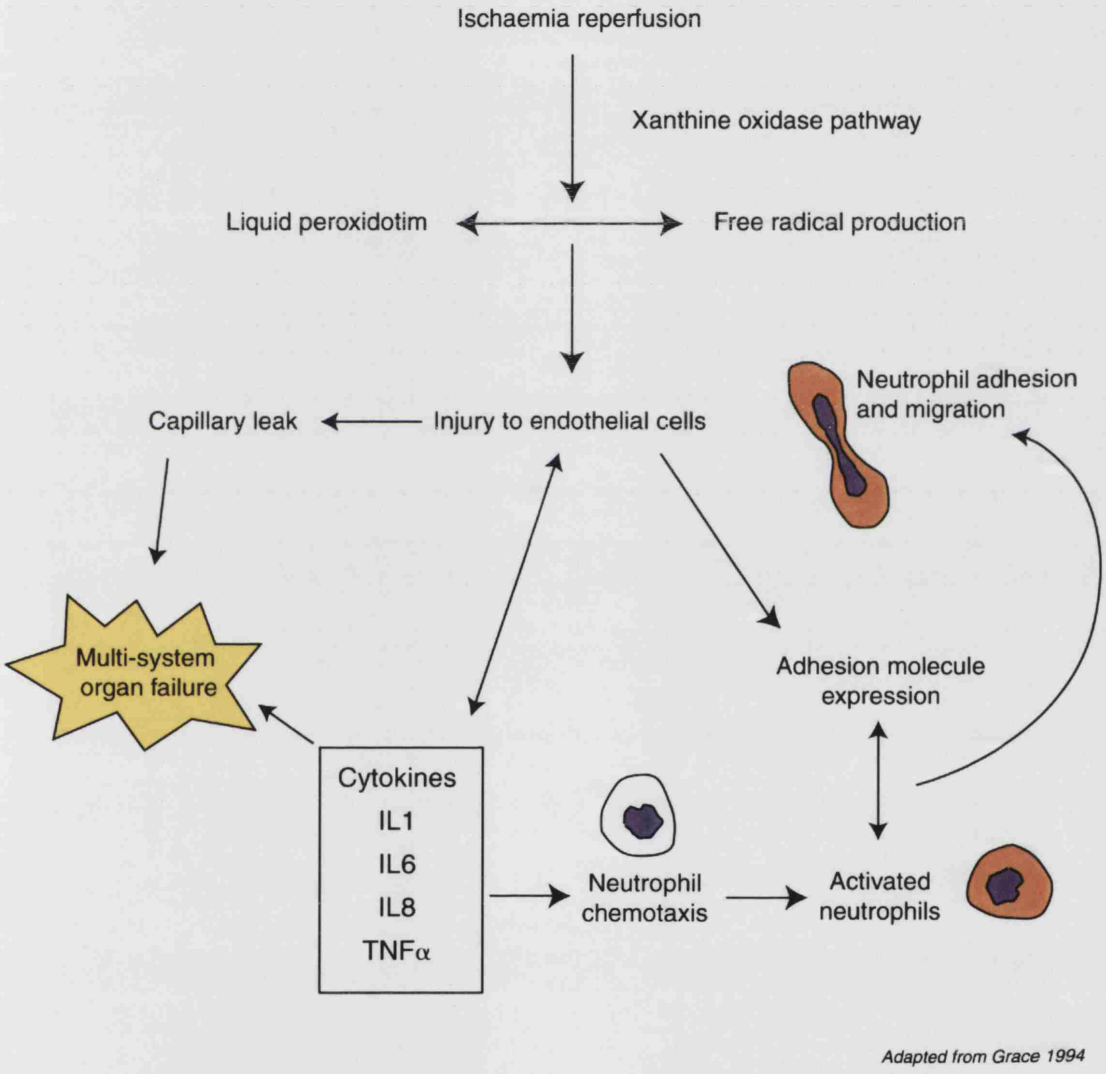
## 6.1 Introduction

Intestinal ischaemia and gangrene are common end points in the cascade of events that result from NEC. The major risk factors for the disease have been discussed in Chapter 1. At the time the stage of intestinal ischaemia and reperfusion (IR) is reached, some of the pathophysiological mechanisms induced, are common to a number of otherwise unrelated intestinal disorders. The clinical spectrum produced is diverse and depends on factors including age at onset, duration of IR and length of affected intestinal segments (DeSa, 1976).

The pathophysiology of intestinal IR is complex and involves damage to local tissue as well as distant organs (Grace, 1994). A number of inter-related mechanisms are involved with interactions that are dependent on the phase of injury (Figure 23). During the ischaemic phase, inadequate tissue oxygenation results in cessation of normal function at a cellular level. Consequent anaerobic metabolism results in a spectrum of injury ranging from oedema and cellular dysfunction to cell death. During re-perfusion, a further sequence of events is initiated. In addition to the

restoration of oxygen carrying capacity, the release of a range of inflammatory mediators takes place. Importantly, neutrophil chemotaxis occurs and reactive oxygen species (ROS) are generated. These have an important role in post reperfusion tissue injury (Kurtel et al., 1991) (Kurtel et al., 1992) which includes increased mucosal permeability and may represent, another central event in the pathogenesis of necrotizing enterocolitis (Langer and Sohal, 1992) (Langer et al., 1993).

**Figure 23** Diagram representing *some* of the complex changes that occur during ischaemia reperfusion.



The timing of surgical intervention is considered important in the effort to reduce morbidity and improve survival for infants with intestinal gangrene. In NEC, the reparative potential of affected bowel is well recognised. As a result, it is widely accepted that surgical intervention is indicated after gangrene is established but before intestinal perforation occurs (Kosloske, 1994b). In practice, determining the optimum time for intervention is often very difficult.

Laparotomy for advanced NEC carries a mortality of 20-50 % (Stringer and Spitz, 1993) (Ade-Ajayi et al., 1996a). The risk of dying from NEC has been linked to the extent of intestinal gangrene (Milner et al., 1986) (Voss et al., 1998) and operative mortality following perforation is thought to double that for necrosis alone (Kosloske, 1985). Despite this imperative, there are few clinical, radiological, haematological, biochemical or microbiological parameters that confidently predict extent of bowel involvement and clinical course in advanced NEC (Dykes et al., 1985) (Kosloske, 1994b). A significant proportion of infants with NEC may have intestinal gangrene without clear evidence.

Available indicators of intestinal ischaemia and gangrene in infants perform inconsistently; cardiovascular instability and persistent metabolic acidosis occur late and are unreliable (Dolgin et al., 1998). Enzyme and metabolic markers that have been reported experimentally and which have been used in the clinical setting include serum amylase, lactic acid, lactate dehydrogenase and phosphate (Jamieson et al., 1979) (De Toma et al., 1983). These tests are reliant on cell death with the release of intra-cellular components which are then reflected in serum assays. Reliable enzyme elevation is therefore almost always a late event.

Specifically, for NEC, considerable effort has been directed at attempts to predict the presence of compromised bowel prior to perforation. Abdominal paracentesis has been advocated (Kosloske, 1994b, Kosloske and Ulrich, 1980), but is invasive and may be difficult to interpret. Novel methods such as recto-sigmoid intra-mural pH assessment, as a means of monitoring splanchnic blood flow are undergoing assessment (Koivusalo et al., 2000) but have yet to be widely validated. A reliable marker for established gangrene would be a major advance in the care of infants under consideration for surgery.

In the work presented in chapter 3, GAGs were shown to occur in all layers of the neonatal intestinal wall using CG histochemistry. HS and CS were predominant with HS largely vascular and CS primarily peri-vascular, in basement membranes and baso-lateral surfaces. GAG disruption to a degree proportionate to NEC severity was demonstrated. Importantly, from the point of view of a potential marker of intestinal pathology, loss of GAGs may consistently precedes major architectural disruption in NEC.

Confluent gangrene is at the extreme end of the spectrum of intestinal disease in NEC and is characterised by an almost complete loss of tissue GAGs. As they are water-soluble and excreted in urine, we hypothesised that intestinal GAGs lost from gangrenous intestine would result in a detectable elevation in urinary excretion of GAGs and that an elevated GAG/Cr would predict the severity of the ischaemic insult.

To examine this hypothesis, we utilised an animal model of NEC and tested urine samples for analysis of the GAG/Cr ratio. In addition we sampled the urine of a group of infants with intestinal ischaemia and compared them to controls. This chapter describes the experiments and reports the results.

## 6.2 Methods

The relationship between the onset of intestinal ischaemia and the timing of surgery cannot be pre-determined in human neonates. An animal model was therefore employed as an adjunct to the human studies to examine the association between duration of IR and resultant urinary GAG levels.

### 6.2.1 Animal studies

The animal models available in NEC have been discussed in Chapter 1. While it is accepted that the IR model is not fully representative of the pathophysiological processes that occur in NEC, it consistently produces the macroscopic and histological changes of NEC. It also provides a robust, reproducible and relatively inexpensive model and as a result, is one of the most widely used. Furthermore, the end point of interest in this experiment was intestinal gangrene.

Ethical approval for this study was granted by the University of Cape Town animal research review committee (Project reference number 96/023). A personal licence was not required.

#### 6.2.1.1 Animals

BD9 rats were admitted to the animal house. Males were used for uniformity. Each animal was weighed and randomly assigned a study number. They were fed and watered *ad libidum* up to and including the morning of surgery when they were assigned to a surgical or control group.

#### 6.2.1.2 Anaesthesia in animals

Anaesthesia was induced by inhalation of ether. Maintenance of anaesthesia and analgesia was achieved using Ketamine; 100mg/kg intramuscularly with additional doses as required. A heating lamp was utilised to prevent hypothermia.

### 6.2.1.3 Surgery in animals

The right groin was exposed in all animals and femoral vein cannulation carried out under direct vision with a 19G Intracath (Deseret ®). Normal saline was delivered by syringe driver (Vial Medical SE 200) intravenously via the femoral catheter at a rate of 20 ml/kg per hour with additional boluses as required to ensure hydration and an adequate urine output for sampling. The bladder was identified and urine specimens obtained by direct aspiration. The urine was frozen prior to being analysed. Blood samples were taken via the femoral line.

Surgery was performed with the aid of a microvascular operating microscope (Nikon SMZ-10). Laparotomy was carried out via a midline incision. Animals in the surgical group had the superior and posterior mesenteric arteries identified and vascular clamps applied. Intestinal ischaemia was confirmed macroscopically. In control animals, a sham laparotomy was carried out with bowel handling only.

### 6.2.1.4 Sampling schedules and termination of experiments

The schedules for sample collection relative to times of ischaemia and reperfusion are given in Table 8. The animals were not recovered and were disposed of by incineration.

### 6.2.2 Studies in human infants

Infants admitted to a neonatal intensive care unit and undergoing laparotomy for NEC were studied. In addition, infants requiring laparotomy for intestinal ischaemia for other reasons and control infants had urine samples collected for analysis (Table 9).

**Table 8 Anaesthesia, surgery and sample collection in rats subjected to IR**

| Animal group | Schedule |
|---|---|
| Surgical (n = 5 ) | Induction of anaesthesia |
| | Laparotomy and intestinal ischaemia |
| | Femoral line insertion |
| | Reperfusion following 30 minutes of ischaemia |
| | Urine sample 2 hours following reperfusion |
| | Blood samples 2 hours following reperfusion |
| Control (n = 4 ) | Induction of anaesthesia |
| | Sham laparotomy |
| | Femoral line insertion |
| | Urine sample 2 hours following sham laparotomy |
| | Blood samples 2 hours following sham laparotomy |

**Table 9 Demographic details and urinary GAGs of infants with intestinal ischaemia**

| Gest. | Sex | Age (days) | Diagnosis | II | GAG/Cr | EP | AHS |
|---|---|---|---|---|---|---|---|
| 32 | m | 12 | NEC | 0 | 86 | CS, (HS) | n |
| 38 | m | 2 | Exomphalos | 0 | 49 | CS | n |
| 40 | m | 113 | Gastroschisis | 0 | 109 | CS | n |
| 40 | f | 6 | Pulmonary HT | *0 | 41 | CS | n |
| 42 | m | 31 | L CDH | 0 | 26 | CS, (HS) | n |
| 35 | m | 6 | NEC | 1 | 82 | CS, HS | y |
| 38 | f | 4 | Malrotation | 1 | 33 | CS | n |
| 25 | m | 8 | Perforated NEC | 1 | 49 | CS, (HS) | n |
| 38 | f | 48 | Multifocal NEC | 1 | 125 | CS, (HS), UC | n |
| 25 | m | 15 | NEC totalis | 2 | 67 | Ins. | Ins. |
| 38 | m | 1 | Volvulus | 2 | 57 | CS, HS, UC | y |
| 28 | f | 15 | NEC totalis | 2 | Inc. | CS, HS, HA | y |
| 30 | f | 8 | NEC totalis | 2 | 54 | CS, HS | y |
| 28 | f | 35 | Colonic NEC | 2 | 68 | CS, (HS) | n |

Abbreviations: Gest., gestational age; II, grade of intestinal ischaemia; GAG/Cr, ratio of quantitative urinary glycosaminoglycans to creatinine; * , intestinal ischaemia score presumed; EP, electrophoresis; CS, Chondroitin sulphate; HS, Heparan sulphate; (HS), trace of Heparan sulphate; UC, unidentified component; HA, Hyaluronic acid; AHS, abnormal Heparan sulphate pattern; n, no; y, yes; NEC, necrotizing enterocolitis; HT, hypertension; L CDH, left congenital diaphragmatic hernia.

#### 6.2.2.1 Urine sampling in human infants

Urine samples were obtained in theatre at the time of laparotomy in all except 2 infants. One in whom it was taken 2 days pre-operatively had NEC totalis at laparotomy. The second was a patient with pulmonary hypertension who did not undergo surgery (Table 9). For infants with a urinary catheter in situ, the sample was obtained from the catheter. Bladder expression by supra-pubic pressure was used to provide urine in infants without a catheter.

### 6.2.3 Urinary assays

#### 6.2.3.1 Urinary assay for animal experiments

In rats, the quantitative measurement of GAGs was carried out as described by Whiteman (Whiteman, 1973a) (for background see Chapter 2). Under controlled pH and electrolyte conditions, Alcian blue added to urine reacts with GAGs, forming insoluble complexes. For these experiments, the complexing agent was 0.05% Alcian Blue in 5 mmol/L magnesium chloride, with 500 mmol/L of sodium acetate buffer at pH 5.8. This was

prepared fresh and spun down, giving a clear blue supernatant. Urine samples were also spun down and 50 µl samples of urine and standard added to 1ml of the complexing reagent in microfuge tubes and allowed to stand for 2 hours. Centrifugation was then carried out at 10,000 rpm (Biofuge Pico Heraens), following which the supernatant was discarded and the precipitate washed twice in absolute ethanol. Next, the Alcian blue - GAG complex was dissolved in 1 ml of 7.5% sodium dodecyl sulphate prior to measurement by spectrophotometry. A standard curve was set up using chondroitin-4-sulphate and absorbance was read at 678nm. Creatinine was measured for each urine sample and a GAG/Cr ratio calculated.

Urinary creatinine was measured on a Beckmam Synchron CX5CE, using a modified rate Jaffe method. Reagents were supplied by Beckman Co.

### 6.2.3.2 Urinary assay for human experiments

In human infants, the quantitative measurement of GAGs was carried out using a modification of the technique described by de Jong (de Jong et al., 1989); urinary cretonne was measured and each urine sample diluted with

distilled water according to the cretonne concentration as shown in Table 10.

Standard and quality control urines were diluted using 0.2 ml of sample and 1.8 ml of water. The working reagent was prepared by adding the dye, 1,9-dimethylmethylene blue (DMB) to Formic acid solution. Trizma base was added to remove interference from urinary protein (de Jong et al., 1992). The diluted urine (0.5 ml) was added to 2.5 ml of working reagent. A colour change was observed (blue to pink), reflecting the formation of complex molecules. This was measured by spectrophotometry. A standard curve was set up using chondroitin-4-sulphate. Following a vortex mix, absorbance was read at 520nm. Duplicate dilutions were made for each sample and if absorbance was above the standard, the test was repeated at a higher dilution. The result was calculated as shown below:

Test absorbance x 200/std. absorbance x 0.2/vol. of urine = GAG mg/L

Result in mg/L / Creatinine in mmol/L = GAG/Creatinine ratio mg/mmol

Urine samples with pH > 8.0 were suggestive of bacterial contamination and therefore considered unsuitable for assay. Age dependent values for the normal range of GAG ratios in humans are given in Table 11.

**Table 10 Dilution of urine based on creatinine concentration**

| Creatinine concentration mmol/L | Volume of urine (ml) | Volume of water (ml) |
|---|---|---|
| **< 0.5** | **1.0** | **1.0** |
| **0.5-1.5** | **0.4** | **1.6** |
| **> 1.5** | **0.2** | **1.8** |

**Table 11 Age dependent glycosaminoglycan to creatinine ratios**

| Age | Mg glycosaminoglycan / mmol Creatinine |
|---|---|
| 0 – 1 month | 10 - 40 |
| 1 - 3 months | 10 - 35 |
| 3 – 6 months | 10 - 30 |
| 6 – 12 months | 5 – 25 |
| 1 – 3 years | 5 - 20 |
| 3 – 5 years | 2 - 15 |
| 5 – 15 years | 2 - 12 |
| > 15 years | 1 - 5 |

### 6.2.3.3 Urine electrophoresis for human experiments

For qualitative analysis, the cellulose acetate sheet was soaked in pyridine/acetic acid buffer, blotted and placed in the tank with the application point 1 cm from the cathode. Wicks were placed over the paper edges and samples applied and allowed to dry.

Electrophoresis was run for 1.5 hours in pyridine/acetic acid as previously described. The sheet was then removed and stained with Alcian blue.

### 6.2.4 Serum assays

Serum amylase, creatinine, and lactate dehydrogenase levels were determined by automated procedures (Department of Biochemistry, Red Cross Children's Hospital, Cape Town).

## 6.3 Results

### 6.3.1 Animal subjects

Nine rats were studied; 5 in the surgical group and 4 controls. The median weight in the control group was 277g (range 227-344) while that in the surgical group was 261 g (range 244 – 311). One rat in the control group had a respiratory arrest secondary to ether inhalational anaesthesia but responded to resuscitation and went on to complete the study.

### 6.3.2 Animal results

There were no significant differences in levels of urine creatinine, plasma creatinine, amylase and lactate dehydrogenase between IR and control groups (Figure 24). Furthermore, the groups could not be distinguished on the basis of the GAG/Cr (Figure 25).

### 6.3.3 Human results

Fourteen infants were sampled. There were 8 boys and 6 girls (Table 9). Six of the infants had NEC with a wide range of clinical severity.

Consistent with our findings in rats, there was no difference in the GAG/Cr when infants with compromised bowel were compared with controls (Figure 26). However, in infants with gangrenous bowel (Figure 27 ), there was a clear increase in the amount of HS compared to CS in all but one (Figures 28 and 29).

**Figure 24** Graphical representation of results of animal experiments comparing ischaemia/reperfusion group with controls for (a) urinary creatinine, (b) plasma creatinine, (c) plasma amylase and (d) lactate dehydrogenase. No differences were demonstrated.

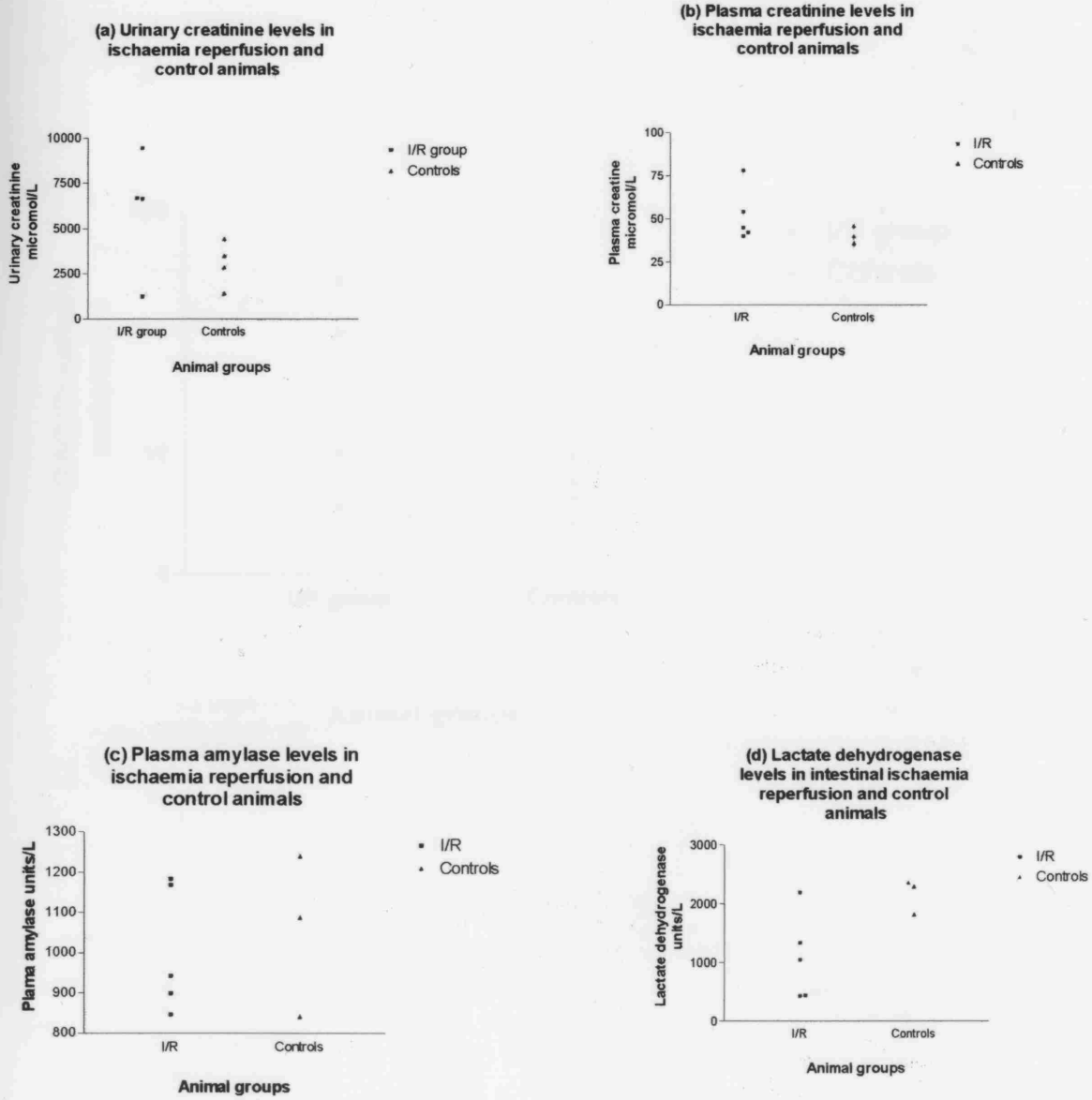


**Figure 25** Graphical representation of results of animal experiments comparing ischaemia/reperfusion group with controls for urinary glycosaminoglycan (GAG) / Creatinine (Cr) ratios. There was no difference between groups.

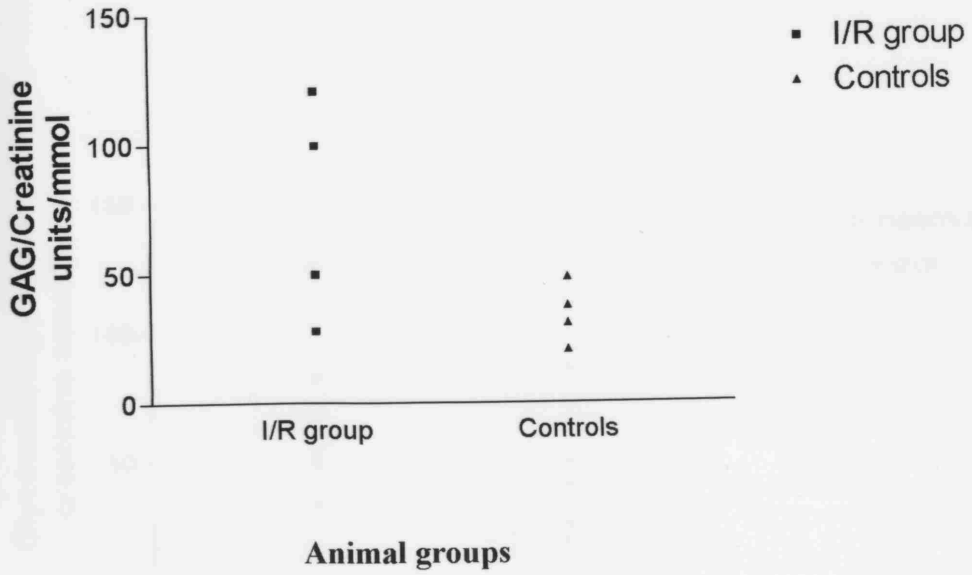


**Figure 26** Graphical representation of results of experiments in human infants comparing intestinal ischaemia group with controls for urinary glycosaminoglycan (GAG) / Creatinine (Cr) ratios. There was no significant difference demonstrated between the groups.

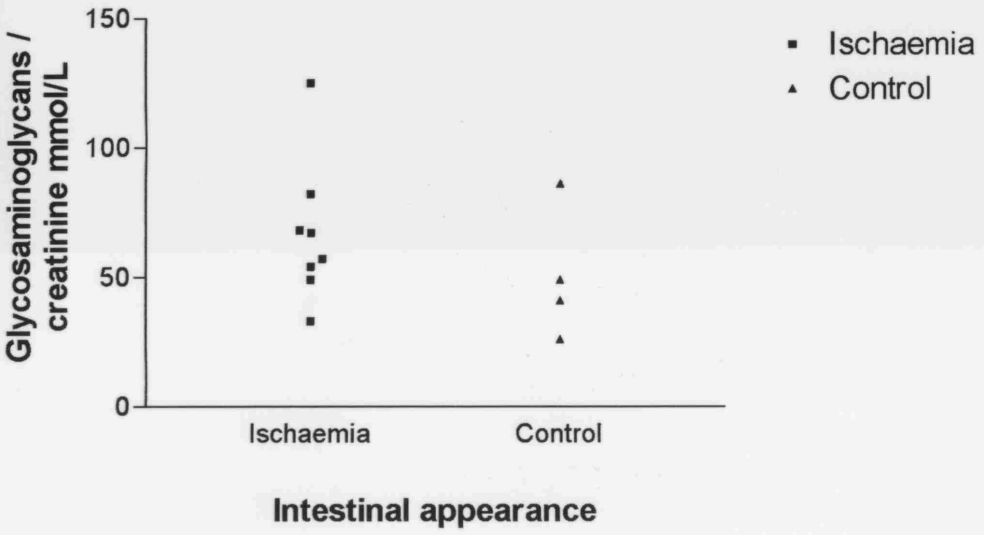
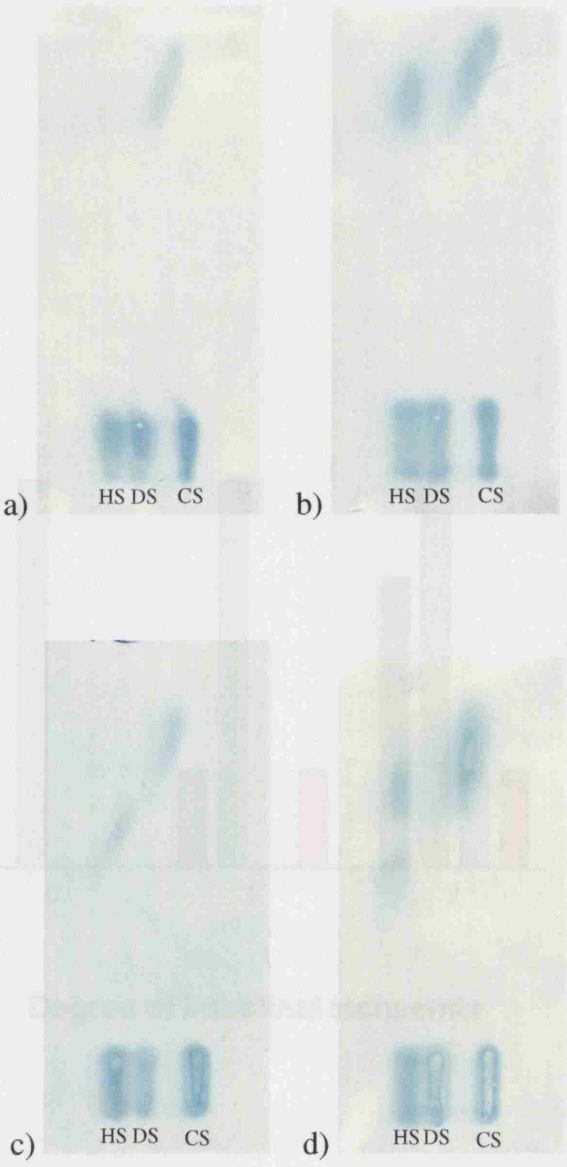


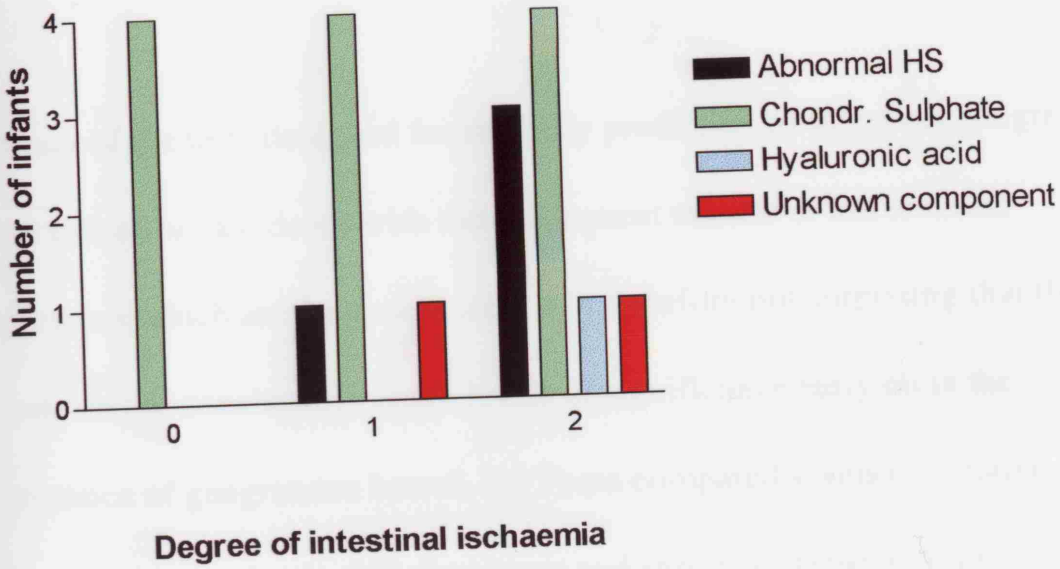
**Figure 27** Infant with confluent intestinal gangrene secondary to neonatal necrotizing enterocolitis.

**Figure 28** Two dimensional cellulose acetate electrophoresis of urine from (a) infant with normal bowel and normal glycosaminoglycan profile, (b) infant with intestinal ischaemia and abnormal profile of Heparan sulphate, (c) infant with unidentified component and (d) infant with gangrenous bowel with abnormal Heparan sulphate profile in addition to unidentified component.




HS, Heparan sulphate; DS, Dermatan sulphate; CS, Chondrotin sulphate, UC, unidentified component.

**Figure 29** Bar chart demonstrating human infants with normal bowel (0), ischaemic bowel (1) and gangrenous bowel (2) and the profile of glycosaminoglycans in the urine.



## 6.4 Discussion

The primary objective of this study was to determine if a GAG / creatinine ratio would reliably predict intestinal gangrene. In both the experimental and human studies, there was a tendency to higher GAG/Cr levels with intestinal ischaemia but this did not reach statistical significance in either group. However, on 2 dimensional electrophoresis there was a striking increase in the density of the HS component of urinary GAGs in infants with intestinal gangrene regardless of cause.

Many of the tests designed for the early prediction of intestinal gangrene are reliant on cell death with the subsequent release of intracellular enzymes which are then measured. It is therefore not surprising that these tests do not consistently reach levels of significance early on in the presence of gangrenous bowel. De Toma compared levels of creatine phosphokinase, lactic dehydrogenase and serum amylase in dogs subjected to acute intestinal ischaemia. Six animals underwent a sham laparotomy and 6, ligation of the superior mesenteric artery. Post operative blood sampling was carried out for 24 hours. Significant increases in serum CPK and LDH were demonstrated in surgical animals compared to controls (De

Toma et al., 1983). We did not demonstrate an increase in the LDH level. This may reflect the fact that our IR experiments were terminated following 30 and 120minutes of ischaemia and reperfusion respectively. A longer period of ischaemia and delayed sampling may be required for the elevation of this and other enzymes which are putative markers of intestinal ischaemia. It is also possible that a longer period of intestinal ischaemia prior to reperfusion would have resulted in a distinction between IR and controls with regard to quantitative GAG/Cr.

The potential strength of urinary GAGs as a marker for intestinal ischaemia lies in the fact that elevation is not reliant on cell death and therefore the observed increase of HS in urine may occur at an early stage. If confirmed, this could prove to be an extremely useful clinical tool.

Interestingly, independent workers into another disease characterised by devastating septic shock and accompanied by CLS, have recently examined urinary GAG excretion as part of an attempt to explain the severity of capillary leak (Oragui et al., 2000). Oragui et al compared urinary excretion of 18 children with meningococcal sepsis with another 18 who had steroid responsive nephrotic syndrome and a further 18 normal

controls. Using polyacrylamide gel electrophoresis and specific enzyme digestion, they detected an increase in the urinary heparan sulphate / creatinine ratio in children with meningococcal septicaema when compared with controls. While direct comparisons cannot be made between these two disease states, it is noteworthy that the CLS is a prominent component of the symptom complex in both and that it is the HS component, the predominant vascular GAG, which is elevated in the urine in the acute phase of both diseases.

## 6.5 Conclusions

In the light of previous attempts to provide reliable evidence of intestinal gangrene, the following conclusions have been drawn from the current study:

- In a rat model of IR in which 30 minutes of ischaemia is followed by 120 minutes of reperfusion, surgical and control groups cannot be distinguished on the basis of urinary or serum creatinine, serum LDH and amylase and urinary GAG/Cr.
- Human infants, with intestinal ischaemia cannot be separated from controls on the basis of quantitative GAGs (overall GAG/Cr).

- There is a definite increase in the density of HS in the urine of infants with intestinal gangrene compared with controls.
- Further studies to quantify the HS elevation and determine consistent threshold for intestinal ischaemia would be valuable.

# Chapter 7

## Summary and conclusions

*7.1. Summary and analysis of preceding chapters*

*7.2. Concluding remarks*

## 7.1 Summary and analysis of preceding chapters

Neonatal necrotizing enterocolitis is a common and complex intestinal disorder with multi-system complications (Kliegman, 1993) (Sonntag et al., 1998) (Morecroft et al., 1994a). Despite extensive scientific investigation, the underlying pathophysiological mechanisms are poorly understood. Clinical presentations are, however, well characterised and two groups of infants may be distinguished on this basis; benign and fulminant. The morbidity and long term mortality in fulminant disease are high (Sonntag et al., 1998).

The work presented in this thesis examines specific pathophysiological mechanisms in NEC and has been influenced by the following observations:

- In the fulminant variety of NEC, the development of CLS appears central to the sequence of events leading to MSOF and death. (Sonntag et al., 1998).
- Modulation of GAGs has been shown to result in CLS in other septic states.

- The attenuation of GAGs has been implicated in protein leakage from the gastro-intestinal tract (Murch et al., 1993) and the congenital absence of GAGs in syndromes in which CLS is a prominent component (Murch et al., 1996) (Westphal et al., 2000).

The primary hypothesis put forward in this thesis was that pathophysiological mechanisms in NEC result in modulation of GAGs. The aims of the project were:

- To ascertain whether there is evidence of altered GAG tissue distribution in the intestine of infants with NEC.
- To examine the relationship between GAG distribution and the inflammatory cell infiltrate in NEC.
- To determine whether there is *in vivo* evidence of GAG excretion in an experimental model and in human infants.

In chapter 3, using gold conjugated poly-L- lysine, the presence of GAGs in all layers of the bowel wall of new-born infants was confirmed. Attenuation of GAGs to a degree proportionate to disease severity was demonstrated. In addition, using Chondroitinase ABC and Heparinase,

vascular GAGs were shown to consist predominantly of HS while matrix GAGs were mostly CS.

The main question arising from these initial results was whether the observed GAG loss was a primary phenomenon related to disease activity or merely a secondary one such as a non-specific response to tissue ischaemia. The data demonstrating graded attenuation with increasing NEC severity suggests the former.

We hypothesised that GAG loss occurs in response to glycanases released as a result of NEC. Cells that produce glycanases include neutrophils, platelets, mast cells and macrophages. Furthermore, the generation of ROS following neutrophil recruitment is well documented (Welbourn et al., 1991) and there is evidence to support their role in the degradation of GAGs (Viscardi et al., 1997). Chapter 4 recorded the results of a further series of experiments undertaken to evaluate the relationship between GAGs and neutrophils and macrophages recruited in the inflammatory response to NEC. A double stain technique using CG for the detection of GAGs and APAAP immunohistochemistry to demonstrate granulocytes was utilised.

The breakdown of endothelial GAGs at the point of neutrophil and macrophage adherence was striking. There was not, however, a spatial correlation between peri-cellular matrix GAG loss and the intensity of neutrophil and macrophage infiltration.

In chapter 5, the panel of monoclonal antibodies was extended to include those against antigens found on lymphocytes, activated endothelial cells and in rapidly dividing cells. In early histopathological NEC, the neutrophils were confined to the serosa. When mucosal ulceration was demonstrated, large numbers of neutrophils and macrophages were then seen in the mucosa.

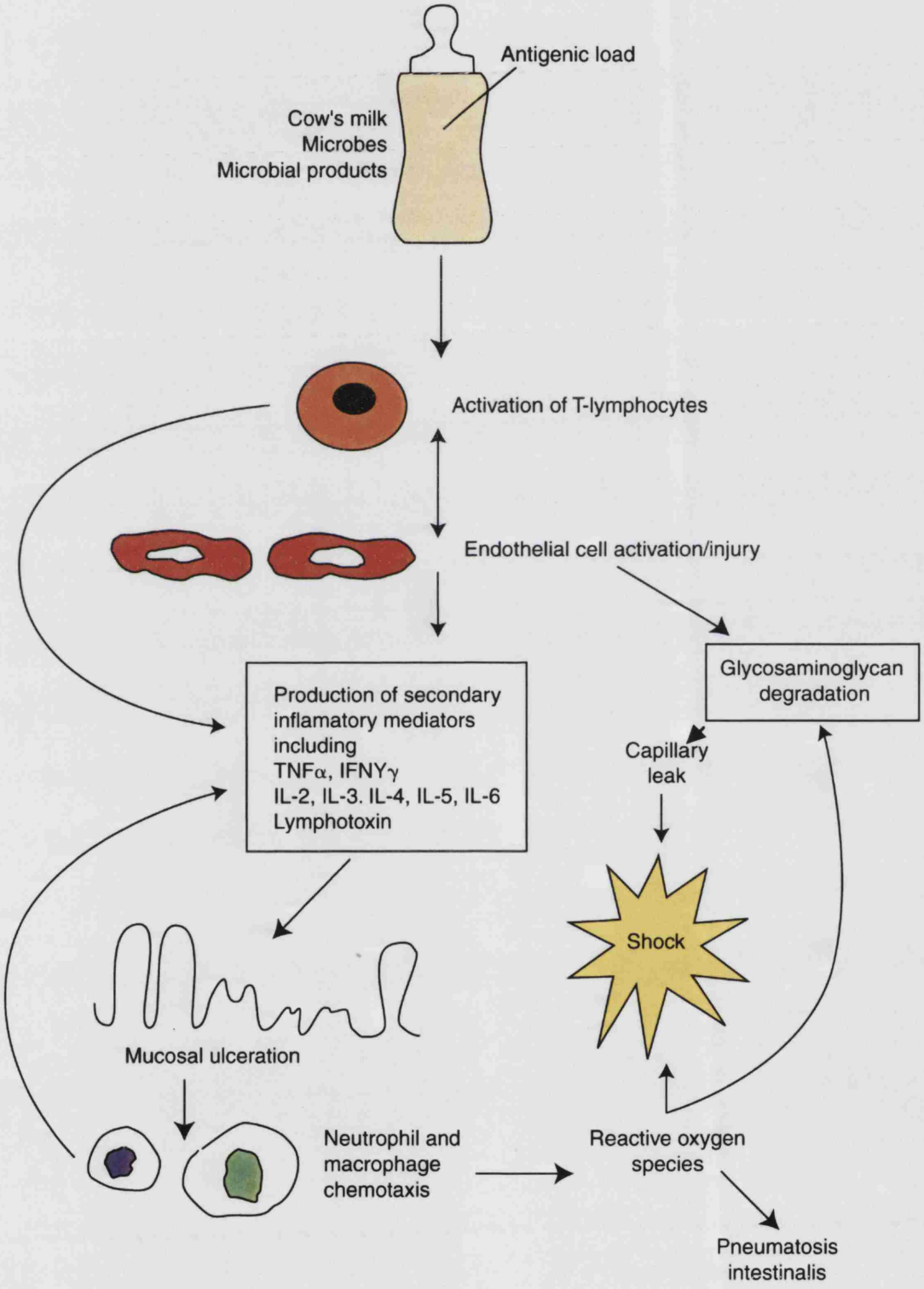
Lymphoid aggregates were prominent and demonstrated a high degree of activation early on in the disease process. While much attention has focused on the role of neutrophils in NEC, our data indicates that lymphocytes also play an early and important role in the inflammatory cascade associated with this condition. Further support for this theory was provided by evidence of endothelial cell activation in submucosal vessels with expression of HLA-DR, ICAM-1, VCAM-1 and E-Selectin. Control

vessels did not express these antigens. The nature of endothelial cell activation is consistent with the presence of lymphocyte derived cytokines such as TNFα and IFNγ. This data supports evidence of complex interactions between a host of inflammatory mediators in NEC previously shown to include PAF, TNF, IFN and iNOS (Muguruma et al., 1997) (Nadler et al., 2000) (Ford et al., 1996). These and other cytokines are involved in neutrophil recruitment and augmentation of the inflammatory response (Viscardi et al., 1997).

T-cell mediated reactions play a role in the gastrointestinal reactions to cow's milk proteins, the expression of which is heterogeneous (Dupont and Heyman, 2000). We suggest that a major pathophysiological component in NEC is a lymphocyte driven, cytokine mediated, intestinal immunopathy in which lymphocyte activation occurs in response to an antigenic load such as cow's milk and bacterial cell walls and their products. Furthermore, it may be that as a consequence of this early activation, mucosal ulceration occurs with influx of large numbers of neutrophils and macrophages. Figure 30 is a representation of the hypotheses examined during this investigation. Putative links between antigenic stimulation and the sequence of events resulting in neutrophil and macrophage chemotaxis in NEC are suggested. These events include lymphocyte activation and the

release of secondary inflammatory mediators resulting in GAG degradation, capillary leak and multi-system dysfunction.

**Figure 30** Hypothetical diagram with mechanisms for the generation and maintenance of fulminant necrotizing enterocolitis.



The above raises the question of whether there may be a role for specific therapeutic inhibition of T cell activation or of T cell derived inflammatory mediators in infants with NEC. In other septic states, the complex biological cascade induced may be effectively attenuated by novel anti-cytokine biotherapies (Moldawer, 1994) (Wherry et al., 1993). Pentoxifylline is a methylxanthine derivative with anti-TNF properties. It has been used by Lauterbach to treat premature septic states (including NEC) with a demonstrable reduction in TNF levels and a reduced mortality in infants on treatment compared with placebo (Lauterbach and Zembala, 1996, Lauterbach et al., 1999). Similar interventions may yet prove to have a wider role in the context of NEC.

In experiments detailed in Chapter 6, we sought to establish whether intestinal GAG attenuation demonstrated in the earlier chapters would result in urinary excretion of GAGs. In particular, we sought to define whether at the worst end of the NEC spectrum, with intestinal gangrene, such excretion had any predictive value. In rat IR experiments we were unable to demonstrate significant differences between the GAG/Cr in surgical animals when compared with controls. Similarly in human infants with intestinal ischaemia, urinary samples taken at the time of laparotomy

did not have significantly elevated GAG/Cr when compared with controls. However, when urine from these infants was subjected to two dimensional electrophoresis, abnormal HS profiles were evident.

## 7.2 Concluding remarks

This thesis provides evidence of GAG modulation in NEC. This has been demonstrated by attenuation of tissue GAGs and an altered profile of urinary GAGs in severe disease. In addition, the distribution of inflammatory cells in different severities of NEC has been demonstrated and a recruitment pattern inferred.

While several questions have been raised by the work presented, further evidence in support of an immunological basis for NEC has been provided. This may contribute to the debate concerning the place of immunologically targeted, pharmacological interventions in NEC. In addition, it is anticipated that future work may establish a role for urinary GAG profiles in the early pre-operative prediction of intestinal gangrene in new-born infants.

**Appendix 1 Modified Bell's criteria for NEC**

| Stage | Classification | Systemic signs | Intestinal signs | Radiologic signs |
|---|---|---|---|---|
| 1a | Suspected NEC | Temperature instability, apnoea, bradycardia, lethargy | Increased gastric aspirate, vomiting, mild abdominal distension, haematochezia. | Normal or intestinal dilatation |
| 1b | Suspected NEC | As for 1a | Bright red blood per rectum | As for 1a |
| 2a | Proven NEC, mildly ill | As for 1a | As for 1b plus absent bowel sounds with or without abdominal tenderness | Intestinal dilatation, ileus, pneu intestinalis |
| 2b | Proven NEC, moderately ill | As for 1a, mild metabolic acidosis, mild thrombocytopenia | As for 2a, definite abdominal tenderness, with or without abdominal wall cellulitis or right lower quadrant mass | Same as for 2a, plus portal vein without ascitis |
| 3a | Advanced NEC, very ill, no intestinal perforation | As for 2b, plus hypotension, bradycardia, apnoea, respiratory and metabolic acidosis, disseminated intra-vascular coagulopathy and neutropenia. | As for 2b, generalised peritonitis, marked tenderness and abdominal distension | As for 2b, definite ascitis |
| 3b | Advanced NEC, very ill, intestinal perforation | As for 3a | As for 3a | As for 3a, pneumoperitoneum |

## Appendix 2 Protocol for dewaxing slides

1. Tissues routinely fixed in formaldehyde and processed in paraffin wax
2. Sections cut at 3microns.
3. Histoclear I x 5 minutes
4. Histoclear II x 5 minutes
5. Take sections down through graded alcohol concentrations;

   100% Ethanol x 3 minutes

   95% Ethanol x 3 minutes

   70% Ethanol x 3 minutes

   50% Ethanol x 3 minutes

   30% Ethanol x 3 minutes
6. Transfer slides into appropriate stain/buffer.

## Appendix 3 Protocol for Haematoxylin and Eosin stain

1. Dewax slides as per Appendix 1.
2. Immerse in Meyer's Haematoxylin for 2-5 minutes.
3. Distilled water for 30 seconds
4. 1% acid alcohol (HCL/70% Ethanol).
5. Gentle running tap water (until blue) - 5 minutes.
6. Distilled water, 30% Ethanol, 50% Ethanol, 70% Ethanol, 95% Ethanol
7. Counterstain with 1% Eosin for 5 mins.
8. Rinse in water.
9. 95% Ethanol
10. 100% Ethanol
11. Histoclear - 10 minutes
12. Histoclear - 10 minutes
13. Mount in DPX (resinous) mountant.

## Appendix 4 Protocol for Poly-L-Lysine stain for GAGs

1. Dewax slides as per appendix 1.
2. Wash in PBS.
3. Apply 5nm gold conjugated poly-L-lysine probe (pH 1.5) to sections.
4. Leave in humidified environment for 1 hour
5. Wash slides in MilliQ
6. Develop stain with silver enhancer x10 minutes.
7. Terminate reaction by washing in tap water.
8. Meyer's Haematoxylin to counterstain x 30 seconds.
9. Mount slides with aqueous mountant (Aquamount Gurr ®).

## Appendix 5 Protocol for APAAP immunohistochemical stain

1. Encircle the section on the slides with a diamond pen.
2. Dewax sections as per appendix 1.
3. Wash off alcohol in TBS.
4. If citrate is required, transfer slides to citrate and microwave for 5 minutes. Allow to sections to cool.
5. Add the appropriate primary mouse monoclonal antibody at 1:50 dilution (in TBS) to sections.
6. Incubate in a moist chamber at room temp for 30 minutes.
7. Tap off the antibody, wash and then place slides in TBS for 5 mins.
8. Add rabbit anti-mouse immunoglobulin at 1:25 dilution (DAKO Code no. Z 259).
9. Incubate in a moist chamber for 30 minutes.
10. Tap off the antibody, wash and then place slides in TBS for 5 mins.
11. Add APAAP complex (DAKO Code no. D651) at a dilution of 1:50.
12. Incubate at room temperature for 30 minutes.

13. Tap off the APAAP complex, wash and then place slides in TBS for 5 mins.

14. For increased staining intensity, repeat steps 7 - 12. Steps 3 and 5 should now be used for 10 minutes only.

15. Add alkaline phosphatase substrate and incubate at room temperature for twenty minutes.

16. Wash in TBS then tap water to terminate reaction. If a double staining with Cationic gold technique is to be used, follow the protocol as per appendix 3.

17. Meyer's Haematoxylin to counterstain x 30 seconds.

18. Mount slides with aqueous mountant (Aquamount Gurr ®).

## Appendix 6 Extravidin Biotin Peroxidase technique

1. Block sections and take to water.
2. Block endogenous peroxidase with 10% Hydrogen peroxide in PBS (20 minutes).
3. Wash in water. Rinse in PBS.
4. Heat mediated antigen retrieval – microwave.
5. Rinse slides in cold PBS.
6. Incubate slides in the appropriate dilution of primary antibody for 60 minutes.
7. Wash slides in PBS – 3 minutes.
8. Incubate slides in the appropriate secondary antibody for 60 minutes; (A) For rabbit polyclonal antisera use biotinylated goat anti-rabbit at 1/200, (B) For mouse monoclonal antisera use biotinylated goat anti-mouse at 1/50.
9. Wash slides in PBS – 3 minutes.
10. Incubate in Extravidin; (A) For rabbit polyclonal antisera – 1/200, (B) For mouse monoclonal antisera – 1/50.
11. Wash slides in PBS – 3 minutes.
12. Develop peroxidase activity with DAB for 10 minutes.
13. Wash in tap water and counterstain with Meyer's haematoxylin.
14. Dehydrate and mount.

## Appendix 7 Protocol for immunohistochemical stains for adhesion molecules

1. Encircle the section on the slides with a diamond pen.
2. Dewax sections as per appendix 1.
3. Wash off alcohol in TBS.
4. Transfer slides to citrate buffer (pH 6) and microwave for 15 minutes.
5. Wash citrate off in water.
6. Add the appropriate primary goat polyclonal antibody at 1:20 dilution
7. Incubate in a moist chamber at room temp for 90 minutes.
8. Tap off the antibody, wash and then place slides in TBS for 5 mins.
9. Add goat antibody at 1:20 dilution.
10. Incubate in a moist chamber for 30 minutes.
11. Tap off the antibody, wash and then place slides in TBS for 5 mins.
12. Add alkaline phosphatase substrate and incubate for twenty minutes.
13. Wash in TBS then tap water to terminate reaction.
14. Meyer's Haematoxylin to counterstain x 30 seconds.
15. Mount slides with aqueous mountant (Aquamount Gurr ®).

## Appendix 8 Protocol for Ki67 immunohistochemical stain

1. Encircle the section with a diamond pen and dewax as per appendix 1.
2. Wash off alcohol in TBS.
3. Add Ki67 antibody at 1:50 dilution (in TBS) to sections.
4. Incubate in a moist chamber at room temp for 30 minutes.
5. Tap off the antibody, wash and then place slides in TBS for 5 mins.
6. Add anti-rabbit immunoglobulin at 1:100 dilution.
7. Incubate in a moist chamber for 30 minutes.
8. Tap off the antibody, wash and then place slides in TBS for 5 mins.
9. Add APAAP complex (DAKO Code no. D651) at a dilution of 1:50.
10. Incubate at room temperature for 30 minutes.
11. Wash and place slides in TBS for 5 mins.
12. Add alkaline phosphatase substrate for twenty minutes.
13. Wash in TBS then tap water to terminate reaction.
14. Meyer's Haematoxylin to counterstain x 30 seconds.
15. Mount slides with aqueous mountant (Aquamount Gurr ®).

## Appendix 9 Isolation of glycosaminoglycans from urine or amniotic fluid.

*Urine samples with pH > 8.0 are unsuitable for analysis.*

*Urine samples with creatinine values < 1.00mmol/l and total GAG < 20mg/l are unsuitable for analysis*

### REAGENTS

Working Alcian Blue Solution

5 ml stock Alcian Blue 1% w/v

2.5 ml 2 mol/l magnesium chloride

10 ml 500 mmol sodium acetate

Make up to 100 ml with distilled water

4M Sodium Chloride

23.4 g sodium chloride in 100 ml distilled water.

0.1M Sodium Carbonate

0.84g sodium carbonate in 100ml distilled water.

Methanol A.R.

Ethanol

## METHOD

For urine only

Add 8 ml working Alcian blue reagent to 2 ml centrifuged urine. Mix and leave to stand either 2 – 4 hours at room temperature or overnight at 4° C. Spin for 10 minutes at 3000 rpm.

For amniotic fluid only

Add 33 ml working Alcian blue reagent to 3 ml amniotic fluid supernatant. Mix and leave to stand overnight at 4° C. Spin for 30 minutes at 3000 rpm.

For both urine and amniotic fluid

Pour off supernatant carefully and blot tube on tissue. Add 200 $\mu$l methanol and mix. Stand for 15 mins mixing a few times to ensure the precipitate is dislodged from the bottom of the tube.

Add 100 $\mu$l 0.1M sodium carbonate and 400 $\mu$l distilled water, mix and stand for 30 min. (May be left at 4° C overnight).

Transfer to 1.4 ml Eppendorf tube and spin for 3 mins. Transfer the clear supernatant to an LP3 tube and add approximately 1.5 ml ethanol, mix and spin for 10 mins at 3000 rpm.

Pour off supernatant carefully and dry precipitate either overnight in air or in vacuum oven at 35° C for 20 mins.

## Appendix 10 Quantitative determination of GAGs in urine using Alcian blue

**Reference: Whiteman P.D. Biochem. J (1973) 131, 343-357**

### Principle

Under controlled conditions of pH and electrolyte conditions, Alcian Blue reacts with glycosaminoglycans (GAGS) to form insoluble complexes. The following procedure has been found to give maximum precipitation of GAGS in standard solutions and urine. Urine addition tests show that up to 100 µl of urine can be added to 1 ml of reagent without significant change in the equilibrium conditions.

### Stock Reagents

Sodium acetate 500 mmol/l

Dissolve 68 g sodium acetate in 800 ml distilled water. Adjust pH to 5.8 with acetic acid A.R. Make up to 1000 ml with distilled water.

Magnesium Chloride 2 mol/l

Dissolve 40 g of magnesium chloride in 95 ml of distilled water. Dilute 1 in 2000.

Alcian Blue Reagent 1% w/v

Dissolve 1 g Alcian Blue in 100 ml distilled water (Alcian Blue 8GX can be obtained from BDH Product No. 34089 Lot No. 2147570L).

Sodium Dodecyl Sulphate (SDS) 75 g/l

Dissolve 15 g sodium dodecyl sulphate (BDH specially pure) in 200 ml distilled water.

Standard

Dissolve 50 mg chondroitin-4 sulphate in 250ml distilled water. Aliquot and store at – 20°C.

**Method**

Set up standard curve in duplicate in LP3 tubes as below.

| **Concentration Mg GAG/1** | **µl Stock STD** | **µl $H_2O$** |
|---|---|---|
| 0 | 0 | 0 |
| 40 | 10 | 40 |
| 80 | 20 | 30 |
| 120 | 30 | 20 |
| 160 | 40 | 10 |
| 200 | 50 | 0 |

Set samples up in duplicate in LP3 tubes. 50 µl urine in each tube.

Add 1 ml working Alcian Blue reagent to all standard and sample tubes. Top and mix.

Incubate at room temperature for 2-4 hrs.

Spin all tubes at 3000 rpm for 15 mins.

Pour off supernatant carefully and blot tubes on tissue.

Add 2 ml absolute ethanol to each tube, top and mix.

Spin all tubes at 3000 rpm for 15 mins.

Pour off supernatant carefully and blot tubes on tissue.

Add 1 ml SDS reagent to each tube and mix. Leave for 30 mins.

Read absorbance at 678 nm.

High samples can be diluted with SDS but better results are obtained by repeating the test using a smaller volume of urine eg 20 $\mu$l rather than 50 $\mu$l.

Subtract blank values from all test and standard values. Plot standard curve. Read mean test values from graph. Results are reported as mg GAG per mmol creat.

*NB. Dilute samples may give falsely elevated results. Samples with a creatinine value <1.0 and GAG <20 mg are reported unsuitable for assay.*

## Appendix 11 Age dependent reference range for quantitative urinary GAGs

| AGE | **MgGAG/mmol creat.** |
|---|---|
| 0 – 1 month | 10 – 40 |
| 1 – 3 months | 10 – 35 |
| 3 – 6 months | 10 – 30 |
| 6 – 12 months | 5 – 25 |
| 1 – 3 years | 5 – 20 |
| 3 – 5 years | 2 – 15 |
| 5 – 15 years | 2 – 12 |
| Over 15 years | 1 – 5 |

## Appendix 12 Urinary GAG electrophoresis

### MATERIALS

Sartorius cellulose acetate sheets type 11200, 78mm x 150mm.

REAGENTS

Pyridine/acetic acid

Add 10 ml conc. Acetic acid (18M) and 100 ml pyridine to water and make volume up to 1000ml.

**M Barium Acetate pH 6.0**

Dissolve 25.5g barium acetate in 1000ml distilled water.

Working Alcian Blue Solution

Take:-

5 ml stock Alcian Blue 1% w/v

2.5 ml 2 mol/l magnesium chloride

10 ml 500 mmol sodium acetate

Make up to 100 ml with distilled water.

(Stock solutions see GAG/Creat. Ratio Worksheet)

**5% Acetic Acid**

Add 50 ml to approx. 1000 ml distilled water.

**METHOD** (See appendix 13 and 14)

## Appendix 13 One dimensional electrophoresis

Mark out cellulose acetate sheet as required using stencil sheet. Soak cellulose acetate sheet in 0.1 M barium acetate, blot and place in tank so that application point is about 1 cm from the cathode side.

Place wicks over edges of paper.

Apply samples and standard, usually 1 ml of each but adjust volume according to concentration of the sample. Allow to dry. Run electrophoresis for approx. 3.5 hrs in 0.1 barium acetate with applied potential 7.5 v/cm. (constant voltage – 50 V. **Always run towards anode and towards "gunge" (ie. Black to red).** Switch off power supply and remove sheet, stain in working Alcian blue reagent for at least 20 mins. Wash at least twice with 5% acetic acid.

## Appendix 14 Two dimensional electrophoresis

Mark out cellulose acetate sheet as required using stencil sheet. Soak cellulose acetate sheet in pyridine/acetic acid buffer, blot and place in tank so that application point is about 1 cm from the cathode side.

Place wicks over edges of paper.

Apply samples, usually 1 ml of each but adjust volume according to concentration of the sample. Allow to dry.

Run electrophoresis for approx. 1.5 hrs in pyridine/acetic acid with applied potential 7.5 v/cm. (constant voltage – 50 V). **Always run towards anode (ie. Black to red).** Switch off power supply and remove sheet, leave in fume cupboard to dry for at least 30 mins.

Cut sheet into two halves, soak both carefully in 0.1 mmol/L barium acetate and blot.

Place sheets in barium acetate tank turning them by 90 degrees ( so that application point is in right hand corner).

Apply standard to each half and allow to dry (bottom right corner).

Run electrophoresis for approx. 3.5 hrs in 0.1 mmol/L barium acetate with applied potential 7.5 v/cm (constant volatge – 50 V). **Always run towards anode and towards "gunge" (ie. Black to red).** Switch off power supply and remove sheet, stain in working Alcian blue reagent for at least 20 mins. Wash at least twice with 5% acetic acid.

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